2006

Functional characterization of human vitamin D receptor residues S237 and R274

Madhuri Dyapa Reddy
San Jose State University

FUNCTIONAL CHARACTERIZATION OF HUMAN VITAMIN D RECEPTOR RESIDUES S237 AND R274

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Madhuri Dyapa Reddy

May 2006

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

Dr. Elaine Collins

Dr. Daryl Eggers

Dr. Roger Terrill

APPROVED FOR THE UNIVERSITY

04/18/06

ABSTRACT

FUNCTIONAL CHARACTERIZATION OF HUMAN VITAMIN D RECEPTOR RESIDUES S237 AND R274

by Madhuri D. Reddy

Crystallographic studies of the vitamin D receptor (VDR) ligand-binding domain show that residues S237 and R274 form hydrogen bonds with the 1-hydroxyl group of 1α,25-dihydroxyvitamin D_3. The cDNA of wild-type or mutant VDR (VDR-S237A, VDR-R274A, VDR-S237A/R274A) was transfected into COS-7 cells or FreeStyle™ 293-F cells to evaluate relative binding affinities. Western blot analysis confirmed that all receptor constructs were expressed as full-length proteins. Binding data indicate that the signal-to-noise ratio was much better with FreeStyle™ 293-F cells than with COS-7 cells. Hormone-binding was undetectable in VDR-R274A and VDR-S237A/R274A mutants. The VDR-S237A mutant bound [^{3}H]-1α,25$(OH)_2D_3$ with approximately 4-fold lower affinity than wild-type VDR. Protease sensitivity assays indicate VDR is more resistant to proteolysis in the presence of ligand. Proteolytic fragment pattern analyses indicate that the mutants stabilize different receptor conformations when bound to hormone. In conclusion, the functional studies confirm the importance of S237 and R274 for proper functioning of VDR.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Elaine Collins, for her guidance, understanding, and constant support throughout my research. I am grateful to Dr. Daryl Eggers for reading a draft of my entire thesis and giving me invaluable feedback. I also wish to acknowledge the immensely helpful feedback from Dr. Roger Terrill.

I am also grateful to all my fellow students and friends for the good times we shared together. I am especially thankful to Ludmila Stoynova for her help, encouragement, and friendship.

Finally, I would like to thank my mother, Dr. D. Chandralekha Reddy, and my father, D. Damodar Reddy, for their unfailing love and encouragement. I am also thankful to my son, Jay, for his love, and my husband, Suresh, for his invaluable help, encouragement, and support throughout my graduate study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Vitamin D is a lipid-soluble vitamin, known for regulation of calcium homeostasis and bone mineralization (Norman, 1979). It was first discovered in connection with a childhood skeletal disease called rickets that occurs when the metabolites of vitamin D are deficient. Two prominent members of the vitamin D group of compounds are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Ergocalciferol is derived from the plant steroid, ergosterol. When the nutritional basis for rickets became apparent, ergocalciferol became a treatment of choice and was employed in the fortification of foods until the 1960s. Cholecalciferol is the form of vitamin D obtained from the conversion of the cutaneous precursor, 7-dehydrocholesterol, in the presence of sunlight when the ultraviolet rays strike the skin. Until the discovery of ergosterol, sunlight was the primary source of vitamin D. In the late nineteenth century, rickets came in an epidemic form when the smoke produced from the factories of the industrial revolution blocked the ultravoilet rays from the sun. Rickets was probably the first childhood disease caused by environmental pollution (Collins and Norman, 2001). By definition, vitamins cannot be synthesized by the body and have to be provided in diet. Since nutritional supplementation is not required in people who have sufficient exposure to sunlight, vitamin D does not meet the classical definition of a vitamin.

The biologically active form of vitamin D is 1α,25-dihyroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], produced by the kidney in response to physiological stimulus (Fraser and Kodicek, 1970; Lawson et al., 1971; Norman et al., 1971; Holick et al., 1971). Like

other steroid molecules, this compound is a derivative of cholesterol; therefore, it is more accurate to classify this compound as a steroid hormone rather than a vitamin (Norman, 1979). The pleiotropic actions of $1\alpha,25(OH)_2D_3$, including calcium homeostasis, cell growth and differentiation, are mediated primarily by its interaction with nuclear vitamin D receptor (VDR), a ligand-dependent transcription factor. VDR is a member of the nuclear receptor superfamily, which includes receptors for glucocorticoids, progesterone, estrogen, aldosterone, androgens, testosterone, thyroid hormone, hormonal forms of vitamin A, and many orphan receptors (Evans, 1988; Lowe et al., 1992).

Conformational Flexibility of Vitamin D Compounds

Vitamin D refers to a group of structurally related compounds that are derived from cyclopentanoperhydrophenanthrene ring system of cholesterol and retain the numbering from the parent steroid compound. Figure 1 shows the numbering and conformational flexibility of vitamin D molecules using $1\alpha,25(OH)_2D_3$ as an example. Vitamin D compounds are classified as secosteroids because the B ring has undergone fission at the 9,10 carbon bond, resulting in only three intact rings and an extended conjugated triene framework connecting the A and C rings. The X-ray crystallographic studies show that the conjugated diene extending between C5 and C8 has *trans* configuration and is nearly planar (Crowfoot and Dunitz, 1948; Hodgkin et al., 1963). However, the conjugated diene system between C6 and C19 has *cis* configuration and is not planar, with the double bond between C10 and C19 twisted out of plane by 60^0. These structural features are a direct consequence of the fission of 9, 10-carbon bond,

which confers conformational mobility to the A-ring to undergo rapid interconversion between two chair conformers (Okamura et al., 1974; Wing et al., 1975). As a result of this interconversion, the A-ring substituents rapidly alternate between axial and equatorial orientations (Figure 1B) (Bouillon et al., 1995). The 360° rotation about the five single carbon-carbon bonds of the side chain results in various positions in the three dimensional space for the C-25 hydroxyl group (Figure 1A) (Okamura, 1992). Also, the 360° rotational freedom about the C6-C7 carbon bond generates conformations ranging from more steroid-like (6-s-*cis*) to the more open and extended (6-s-*trans*) conformation of the molecule (Figure 1C) (Okamura, 2000). This unusual conformational flexibility is unique to vitamin D molecules in comparison with the rigid structures of other steroid hormones (Collins and Norman, 2001). It is supposed that the different shapes generated by the conformationally flexible hormone enable it to effectively adapt to the ligand binding domains of nuclear VDR, vitamin D binding protein, and the putative membrane VDR (Norman et al., 1996).

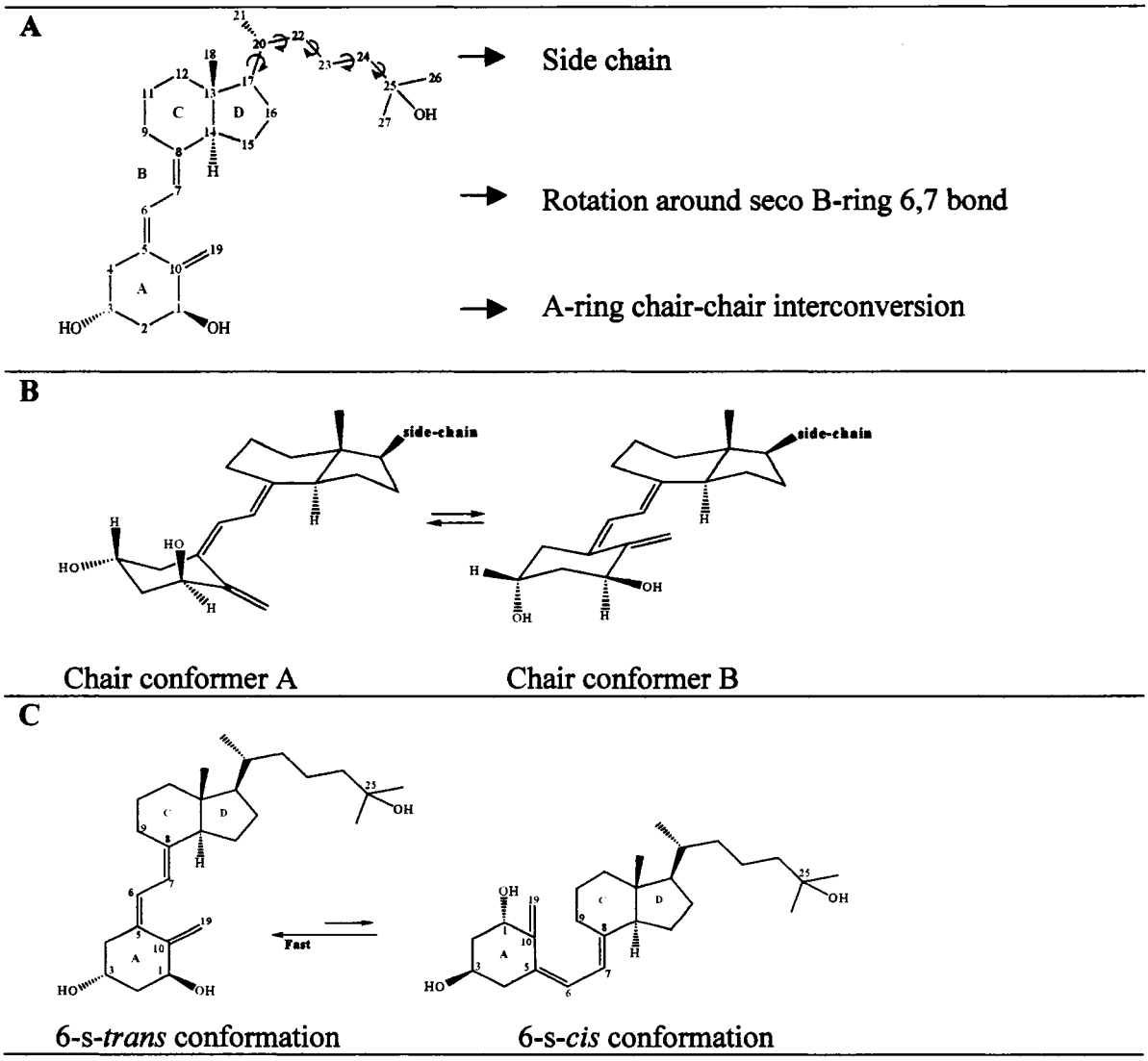


Figure 1. Conformational flexibility of 1α,25(OH)$_2$D$_3$. **(A)**: Structure of 1,25(OH)$_2$D$_3$. **(B)**: Two conformations of A ring as a result of rapid chair-chair interconversion. Chair conformer A has 1α-OH and 3β-OH groups in axial and equatorial orientations, respectively, whereas chair conformer B has 1α-OH in equatorial and 3β-OH in axial orientations. **(C)**: Conformations ranging from steroid-like (6-s-*cis*) to extended (6-s-*trans*) conformation due to the 360° rotational freedom of 6,7 bond of the seco B ring.

Metabolism

Two sources of vitamin D are diet and cutaneous synthesis. The vitamin D derived from vegetable sources is vitamin D_2, and that from animal sources is vitamin D_3. These two forms are metabolized identically, and both have been used to fortify foods. Although vitamin D can be obtained from diet, the major source of vitamin D_3 production is from the nonenzymatic photoconversion of 7-dehydrocholesterol, present in the skin, to a secosteroid previtamin D_3. The previtamin D_3 then isomerizes to vitamin D_3 over a period of several days (Holick, 1981). Alternatively, previtamin D_3 can isomerize to inert byproducts, tachysterol and lumisterol, thus preventing production of excessive amounts of vitamin D_3 with prolonged exposure to sun. The degree of skin pigmentation also regulates production of vitamin D_3 by blocking the penetration of ultraviolet rays. Once formed, vitamin D_3 enters the circulatory system where it binds to vitamin D binding protein (DBP). Also known as a group-specific component (Gc), DBP is a 52 kDa, highly expressed multifunctional serum protein (White and Cooke, 2000). Besides serving as a transport protein for vitamin D and its metabolites (Daiger et al., 1975), DBP is a very effective actin-binding protein (Van Baelen et al., 1980; Otterbien et al., 2002). As such, DBP plays a crucial role in the actin-scavenger system responsible for rapid depolymerization and clearance of actin filaments from the bloodstream (Haddad et al., 1990; Lee and Galbraith, 1992; White and Cooke, 2000) that otherwise have lethal consequences. In addition to these functions, DBP is also associated with macrophage activation (Yamamoto and Homma, 1991) and chemotaxis (White and Cooke, 2000).

Vitamin D_3 is largely biologically inert. In order to exhibit biological activity, it

has to undergo two sequential hydroxylations, first at carbon-25 in the liver and then at carbon-1 in the kidney to form $1\alpha,25(OH)_2D_3$, the most active metabolite of vitamin D_3 known. Another dihydroxylated metabolite of vitamin D_3, 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], is also reported to possess biological activity (Henry and Norman, 1978; Norman et al., 1983). Vitamin D_3, bound to DBP, is transported to the liver where vitamin D_3-25-hydroxylase catalyzes the conversion of vitamin D_3 to 25-hydroxyvitamin D_3, an inactive metabolite. Vitamin D_3-25-hydroxylase, present in the liver microsomes and mitochondria (Saarem et al., 1984; Axén et al., 1994), is a cytochrome P450-like enzyme that is poorly regulated (Tucker et al., 1973). The major circulating reserve form of vitamin D is the 25-hydroxyvitamin D_3, which defines the vitamin D status of an individual (Pettifor et al., 1977; Mawer, 1980).

From the liver, the 25-hydroxyvitamin D_3 again enters the circulatory system, binds to DBP, and is then carried to the kidney. In the kidney, 25-hydroxyvitamin D_3-1-α-hydroxylase (1-hydroxylase) enzyme adds the second hydroxyl group at the carbon-1 position to form the hormonally active $1\alpha,25(OH)_2D_3$ (Henry and Norman, 1974). The 1-hydroxylase enzyme belongs to a class of enzymes known as mixed-function oxidases and is cytochrome P450 dependent (Ghazarian et al., 1974). The activity of 1-hydroxylase is inversely related to the vitamin D status of an animal. The renal production of $1\alpha,25(OH)_2D_3$ is modulated by the levels of $1\alpha,25(OH)_2D_3$ itself, parathyroid hormone (PTH), and plasma concentrations of calcium and phosphate (Henry et al., 1974). When serum calcium levels are low, PTH is released, stimulating the renal synthesis of $1\alpha,25(OH)_2D_3$ by activating the 1-hydroxylase enzyme (Jones et al., 1998).

Synthesis of $1\alpha,25(OH)_2D_3$ leads to an increase in serum calcium levels largely by increasing the efficiency of intestinal absorption of calcium. When the plasma calcium levels reach the physiological range of 9.5-10.5 mg/100 mL (Norman, 1979), $1\alpha,25(OH)_2D_3$ decreases the transcription of the PTH gene, resulting in decreased PTH secretion (Silver and Kronenberg, 1996). There is also evidence of regulation of calcium and phosphate levels by $1\alpha,25(OH)_2D_3$ in a PTH-independent manner, which appears to be mediated by changes in mRNA levels of 24-hydroxylase and 1-hydroxylase genes (Weisinger et al., 1989; Shinki et al., 1992).

The second dihydroxylated metabolite of vitamin D_3 produced in the kidney is $24,25(OH)_2D_3$. Some of the biological actions attributed to $24,25(OH)_2D_3$ include the following: mineralization of bone, fracture healing, and a role in suppression of PTH secretion. Research has shown that the combined presence of both the dihydroxylated metabolites, $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$, are required for normal egg production, fertility, and hatchability in chicken (Henry and Norman, 1978). The enzyme that catalyzes the formation of $24,25(OH)_2D_3$ from 25-hydroxyvitamin D_3 is 25-hydroxyvitamin D_3-24-hydroxylase (24-hydroxylase), present in renal mitochondria. The PTH stimulation of the activity of 1-hydroxylase in response to hypocalcemia is accompanied by a decrease in activity of 24-hydroxylase. Conversely, when the levels of $1\alpha,25(OH)_2D_3$ are high, renal expression of 1-hydroxylase is decreased and 24-hydroxylase levels are increased.

In addition to the aforementioned metabolites of vitamin D_3, there are 35 chemically characterized metabolites, most of which are intermediates in the degradation

pathways of $1\alpha,25(OH)_2D_3$ and have no known biological activity. One exception is the renal production of $1\alpha,25(OH)_2D_3$-26,23-lactone (Ishizuka, 1988). This metabolite is associated with very high plasma levels of $1\alpha,25(OH)_2D_3$ and is believed to inhibit the resorptive actions of $1\alpha,25(OH)_2D_3$ on bone. Excretion of vitamin D and its metabolites occurs primarily in the feces with the aid of bile salts, and very little appears in the urine (Collins and Norman, 2001).

Mechanism of Action

Many of the biological actions of $1\alpha,25(OH)_2D_3$ are mediated by binding to the nuclear VDR. However, similar to other steroid hormones such as estrogen (Morley et al., 1992), testosterone (Koenig et al., 1989), progesterone (Mendoza and Tesarik, 1993; Wistrom and Meizel, 1993; Blackmore et al., 1990; Majewska and Vaupel, 1991; Mendoza and Tesarik, 1993), thyroid hormone (Smith et al., 1992; Segel, 1990), glucocorticoids (Rehberger et al., 1992; Gametchu et al., 1993) and corticosteroids (Orchinik et al., 1991), there are some biologic effects attributed to $1\alpha,25(OH)_2D_3$ that are too rapid for transcriptional mechanisms (Nemere and Norman, 1987). Thus, physiological actions of $1\alpha,25(OH)_2D_3$ can be classified as those that are mediated by nuclear VDR that are genomic responses and those that are mediated by a putative membrane receptor that are rapid nongenomic responses.

Actions Mediated by Nuclear VDR: $1\alpha,25(OH)_2D_3$ functions similarly to other steroid hormones. A schematic representation of the mode of action of $1\alpha,25(OH)_2D_3$ is shown in Figure 2. In response to a physiologic stimulus, $1\alpha,25(OH)_2D_3$ is produced by the

kidney and enters the circulation. In the plasma, the hormone binds to the carrier protein, DBP, and is transported to target cells. Being a lipid-soluble molecule, $1\alpha,25(OH)_2D_3$ can easily penetrate the plasma membrane of the target cell. Once the hormone enters the nucleus of the target cell, it binds to the specific, high-affinity VDR. The hormone-VDR interaction induces conformational changes in the receptor that includes formation of a hydrophobic cleft on the receptor surface due to the repositioning of helix 12 (Norman et al., 1999; Rochel et al., 2000). Formation of the active hormone-receptor complex is also thought to involve phosphorylation of the VDR (Haussler et al., 1988; Jurutka et al., 1993; Darwish et al., 1993a). Phosphorylation could increase affinity of VDR to coactivators, which are molecules recruited by transcription factors to enhance their transcriptional activity. The altered receptor conformation facilitates heterodimerization with the unliganded retinoid X receptor (RXR), interaction with coactivators, and the basal transcriptional machinery to form a transcriptionally active complex (Nayeri and Carlberg, 1997). In this active conformation, the receptor recognizes specific sequences on the DNA called hormone response elements that are present in the promoter regions of target genes, and initiates regulation of transcriptional activation or repression of hormone sensitive genes (Hannah and Norman, 1994; Haussler et al., 1997). The modulation of gene expression results in the changes in protein expression that are needed to produce the required physiological responses.

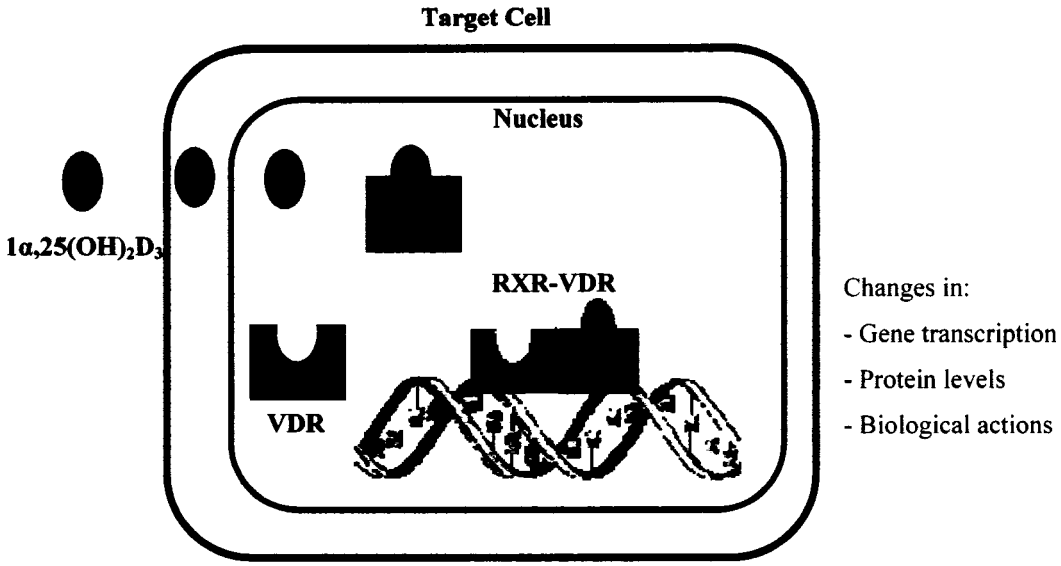


Figure 2. Mode of action of $1\alpha,25(OH)_2D_3$.

Rapid Responses Mediated by the Putative Membrane Receptor for $1\alpha,25(OH)_2D_3$: The nongenomic actions of $1\alpha,25(OH)_2D_3$ include rapid intestinal transport of calcium, activation of signal transduction pathways such as protein kinase C (PKC) (Bissonnette et al., 1994; Khare et al., 1994; Slater et al., 1995), and opening of voltage-gated calcium channels (Caffery and Farach-Carson, 1989). These actions are seen in various cell types within minutes of exposure to the hormone. The organs and cell locations of rapid responses include intestine (DeBoland et al., 1990; Nemere et al., 1984; DeBoland and Norman, 1990; Zhou and Norman, 1992), liver (Baran et al., 1989; Baran et al., 1990), muscle, and osteoblasts (Meikle, 1988). Rapid hormonal stimulation of calcium absorption in the intestine, termed transcaltachia, is not inhibited by the genomic inhibitor actinomycin D, nor is it inhibited by the protein synthesis inhibitor, cycloheximide.

However, nifedipine, a calcium channel blocker, H7, an inhibitor of phospholipase C, and U73122, a PKC inbibitor, have inhibitory effects on transcaltachia. Mastoparan, which activates G proteins, stimulates transcaltachia (DeBoland et al., 1990; Nemere et al., 1984; DeBoland and Norman, 1990; Zhou and Norman, 1992). These data support the hypotheses that the rapid actions of $1\alpha,25(OH)_2D_3$ are mediated by cell membrane and cytoplasmic components (Nemere et al, 1984; Norman et al., 1984). Based on these findings, a putative cell membrane receptor called mVDR has been proposed for $1\alpha,25(OH)_2D_3$. The mVDR initiates a variety of signal transduction pathways independent of activation of gene transcription (Nemere et al., 1994). The complete cDNA of a unique membrane receptor for $1\alpha,25(OH)_2D_3$, unrelated to the nuclear VDR, has been published and named $1,25D_3$-MARRS (membrane-associated, rapid response, steroid-binding protein) (Nemere et al., 2004). This receptor is associated with $1\alpha,25(OH)_2D_3$-dependent regulation of phosphate uptake in chick intestinal epithelial cells. However, recent studies using a VDR knockout mouse (Zanello and Norman, 2004) or a naturally occurring human VDR mutation in the DNA binding domain, K45E (Nguyen et al., 2003), have shown that a fully functional nuclear VDR is required for $1\alpha,25(OH)_2D_3$-mediated rapid responses.

Nuclear Vitamin D Receptor

The nuclear receptor for $1\alpha,25(OH)_2D_3$, VDR, is a ligand-inducible transcription factor. The cDNA for VDR was first cloned from chicken in 1987 (McDonnell et al., 1987) and shortly thereafter from human (Baker et al, 1988). The domain structure for

VDR is similar to other nuclear receptors and consists of four domains (Krust et al., 1986) that are modular in nature (Green and Chambon, 1988; Ham and Parker, 1989). Figure 3 outlines the functional domains of VDR.

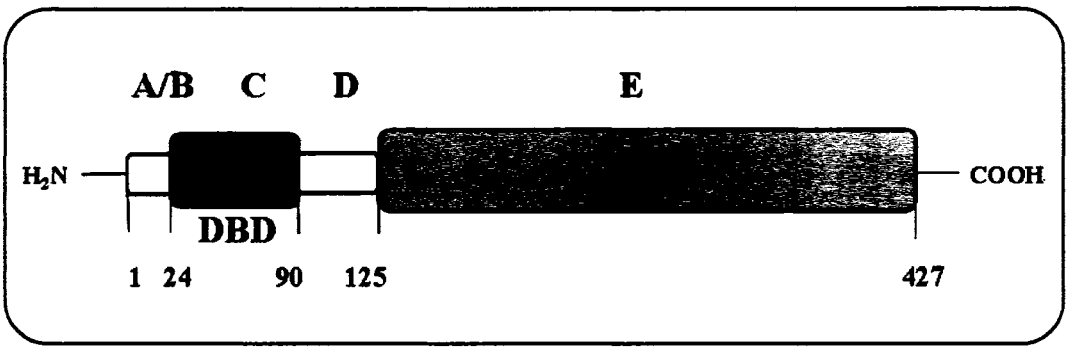


Figure 3. Domain structure of the VDR.

DNA Binding Domain (DBD), C: This is the most conserved domain among nuclear receptors. It contains about 66 amino acid residues that fold into two zinc finger-like structures that allow the receptor to interact with specific sequences on DNA, called hormone response element (HRE), present in the promoter region of target genes. Each zinc ion is held in a tetrahedral arrangement by conserved cysteine residues. The first zinc finger, which contains four cysteine residues and several hydrophobic amino acids, is critical for determining DNA target site selectivity. The second zinc finger, which contains five cysteine residues and many basic amino acid residues, mediates protein-protein interactions involved in dimerization (Evans, 1988; Green and Chambon, 1988; Forman and Samuels, 1990; Rastinejad et al., 1995). The HRE sequence is a direct

repeat (DR) of 5'-AGGTCA-3' for the $1\alpha,25(OH)_2D_3$, thyroid (TR) and retinoic acid (RAR) receptors (Kerner et al., 1989; Demay et al., 1990; Carlberg et al., 1993; Schule et al., 1990). In VDR, a 3-base pair spacer separates the two hexameric half-sites and fixes their relative orientation, while that of TR and RAR prefer four (DR-4) and five (DR-5) base pair spacers, respectively (Umesono et al., 1991). The spacers are not only important for determining receptor preference (Collins, 2000) but also for high affinity binding of RXR-RAR, RXR-TR, and RXR-VDR heterodimers (Umesono et al., 1991; Mangelsdorf and Evans, 1995), with RXR occupying the upstream half-site in each element (Shaffer and Gewirth, 2002).

Ligand Binding Domain (LBD), E: This domain is the next most conserved region after DBD. It contains 302 amino acid residues located at the C-terminal end of the receptor. The overall topology of this domain in VDR is similar to LBDs of other nuclear receptors. The domain is composed of a three-stranded β-sheet and 12 α-helical structures that fold to provide a hydrophobic ligand binding pocket (Norman et al., 1999; Rochel et al., 2000). In addition to steroid binding, this domain also functions in receptor dimerization, nuclear translocation and hormone-dependent transcriptional activation. Ligand binding-induced repositioning of H12, containing activation function (AF-2) domain, is critical for interaction with coactivators.

Hinge Region, D: Between the DBD and the LBD is the flexible hinge region. It consists of 35 amino acids and has immunogenic properties. This region provides flexibility to both DBD and LBD for proper orientation. Residues 97-121 within the hinge region form a long helical structure similar to that seen in the hinge region of thyroid receptor

(Rastinejad et al., 1995). Studies with VDR mutants containing deletions of the internal residues in the hinge region of VDR have indicated that, despite retaining the ability to form heterodimers and bind response elements with high affinity, a full-length hinge region is essential for retention of transcriptional activity (Shaffer et al., 2005).

A/B Domain: Present at the N-terminal end of the receptor is the A/B domain, which is the least conserved domain among nuclear receptors. Although the function of this domain in VDR is not clearly defined, in other nuclear receptors it is known to have immunogenic properties. The A/B domain, comprised of only 24 amino acid residues, is shortest in VDR in comparison with the A/B domains of other members of the nuclear receptor superfamily. This domain in VDR lacks the independent transcriptional activation function (AF-1) domain that is constitutive in receptor constructs lacking LBD (Aranda and Pascual, 2001). The removal of this domain does not appear to affect hormone binding, DNA binding, or the transactivation properties of VDR (Issa et al., 1998).

VDR-Ligand Interaction

Crystallographic studies of the LBD of VDR docked with $1\alpha,25(OH)_2D_3$ (Rochel et al., 2000) have provided key structural insights into the nature of the ligand-receptor interaction. Unique to LBD of VDR, in comparison with other nuclear receptors, is the presence of a large, flexible insertion domain of 72-81 residues between helices 1 and 3 that prevents the formation of stable crystals. So, the crystal structure of LBD lacking the insertion domain residues 165-215 in a complex with $1\alpha,25(OH)_2D_3$ was determined.

This deletion does not appear to have any major effect on ligand binding, dimerization with RXR, and transcriptional activity (Rochel et al., 2000).

The existence of large conformational differences between apo- and holo-receptors suggests that ligand binding induces structural changes within the LBD (Peleg, 1995). The most obvious conformational change observed following hormone binding is the repositioning of helix 12, containing the AF-2 domain. In the apo-conformation, H12 is extended downward creating an open portal for $1\alpha,25(OH)_2D_3$ to gain access to the ligand binding cavity. Once the hormone enters the ligand binding pocket, the repositioning of helix 12 upward to interact with helices 3 and 5 closes the portal and creates a hydrophobic cleft on the protein surface that is important for interaction with coactivator proteins.

The ligand binding cavity in VDR is large (697 $Å^3$) and can easily accommodate the conformationally flexible $1\alpha,25(OH)_2D_3$ that occupies about 56% of the cavity (Rochel et al., 2000). The residues lining the ligand-binding pocket are predominantly hydrophobic. Within the cavity, the A-ring of the hormone is oriented toward the C-terminus of helix 5 and assumes the chair B conformation, with 1-hydroxyl group in equatorial and 3-hydroxyl group in axial orientations. The ligand adopts a curved shape in the hydrophobic pocket because of the nonplanar geometry of the conjugated triene connecting the A and C rings. The side chain of the ligand is oriented toward helix 11 where the cavity is widest. Within the hydrophobic pocket the hydroxyl groups of the ligand are in contact with polar residues (Figure 4). The biologically important 1-hydroxyl group of $1\alpha,25(OH)_2D_3$ forms two hydrogen bonds with S237 and R274,

present in helices 3 and 5, respectively. In addition to forming a hydrogen bond with the hormone, R274 also forms hydrogen bonds with water molecules forming a water channel. The 3-hydroxyl group of $1\alpha,25(OH)_2D_3$ forms two hydrogen bonds with S278 in helix 5 and Y143 in helix 1, while the 25-hydroxyl group forms hydrogen bonds with H305, present in the loop H6-H7, and with H397 in helix 11. Site-directed mutagenesis, targeting residues that contact the hydroxyl groups of the ligand, has shown that R274 and H397 play a critical role in ligand binding, since mutations R274A and H397A have a significant effect on ligand affinity as well as transcription potency. Mutational analyses have also shown that S237 and H305 have an assistant role in binding (Yamada et al., 2003), while the contribution of Y143 and S278 is only minor (Yamada et al., 2003; Acevedo et al., 2004), since mutations of these residues have less effect than those of S237 and H305.

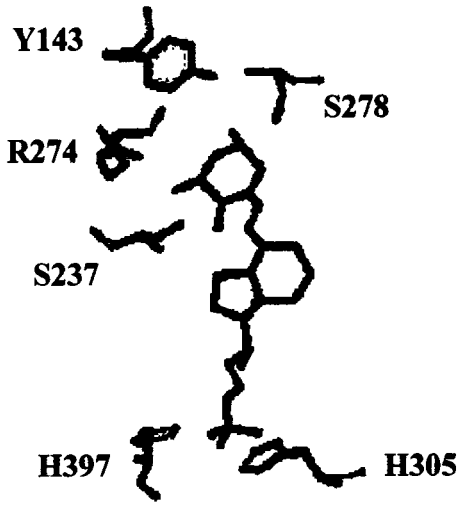


Figure 4. Polar residues within the hydrophobic pocket that contact the hydroxyl groups of 1α,25(OH)$_2$D$_3$. Residues in the ligand binding domain that form hydrogen bonds with the three hydroxyl groups of 1α,25(OH)$_2$D$_3$ ligand are shown. Oxygen atoms are colored in red, nitrogen atoms in blue, and carbon-carbon bonds in grey.

Physiological Actions of 1α,25(OH)$_2$D$_3$

Approximately 26 target organs contain VDR and more than 50 genes, including several oncogenes, are known to be regulated by 1α,25(OH)$_2$D$_3$ (Collins, 2000). As mentioned earlier, 1α,25(OH)$_2$D$_3$ exhibits a variety of physiological actions mediated by nuclear VDR that are genomic in nature. In addition there are several rapid responses attributed to 1α,25(OH)$_2$D$_3$ that are thought to be mediated through nongenomic signal transduction pathways.

Intestinal Calcium Absorption: Three pathways, namely, transcellular route, vesicular calcium transport, and paracellular transport, achieve absorption of calcium by the

intestine. Absorption by transcellular route and vesicular transport are mediated by $1\alpha,25(OH)_2D_3$. Transcaltachia via the vesicular pathway involves nongenomic actions of the hormone (Nemere, 1992). In this model, calcium enters the cell either through a calcium channel or by endocytosis and localizes into lysosomes; calcium from the lysosomes is extruded from the cells by exocytosis. There is evidence that a calcium-binding protein, calbindin-D28K, is involved in this process (Nemere et al., 1986). Although the necessity of $1\alpha,25(OH)_2D_3$ for paracellular calcium absorption remains controversial (Fleet and Wood, 1999), there is substantial evidence that the hormone can enhance paracellular calcium absorption (Karbach, 1992; Kutuzova and DeLuca, 2004). The transcellular route is the most extensively studied mechanism of intestinal calcium absorption. Entry of calcium into the enterocyte is regulated by various brush border proteins, including intestinal membrane calcium binding protein (Schachter and Kowarski, 1982) and brush-border alkaline phosphatase, both of which are induced by $1\alpha,25(OH)_2D_3$. Once calcium enters the enterocyte, it preferentially binds a $1\alpha,25(OH)_2D_3$-inducible protein, calbindin-D9K that associates with microtubules and may play a role in the transport of calcium across the enterocyte. The final step involved in intestinal calcium absorption is the extrusion of calcium from the antiluminal surface of enterocyte into the circulation; this mainly involves a $1\alpha,25(OH)_2D_3$-inducible ATP-dependent calcium pump (Bronner et al., 1986).

Actions on Parathyroid Gland: Regulation of transcription of parathyroid hormone gene by $1\alpha,25(OH)_2D_3$ has been reported for studies both *in vitro* (Russel et al., 1986) and *in vivo* (Silver et al., 1986). Physiological doses of $1\alpha,25(OH)_2D_3$ increase expression of

VDR gene in the parathyroid glands and decrease mRNA levels in PTH (Naveh-Many et al., 1990), indicating that $1\alpha,25(OH)_2D_3$, acting through its receptor, suppresses preparathyroid hormone gene transcription. This action has been exploited in the therapeutic use of $1\alpha,25(OH)_2D_3$ for treatment of hyperparathyroidism associated with chronic renal failure. In addition, the steroid hormone has an inhibitory effect on the proliferation of dispersed parathyroid cells in culture (Canalejo et al., 2000).

Actions on Bone: $1\alpha,25(OH)_2D_3$ has numerous effects on bone. It regulates transcription of two of the most abundant proteins present in the bone matrix; it represses the synthesis of type 1 collagen (Harrison et al., 1989; Rowe and Kream, 1982) and induces the synthesis of osteocalcin (Pan and Price, 1984). The hormone provides the proper microenvironment for bone mineralization through stimulation of intestinal absorption of calcium and phosphate. Although a powerful antirachitic agent, $1\alpha,25(OH)_2D_3$ stimulates bone resorption (Collins, 2000). Receptors for $1\alpha,25(OH)_2D_3$ are present in osteoblastic cells (Walters et al., 1982) and not present in the osteoclasts. The hormone promotes the differentiation of osteoclasts from monocyte-macrophage stem cell precursors *in vitro* (Roodman et al., 1985). High doses of $1\alpha,25(OH)_2D_3$ stimulate the production of osteoclast-differentiating factor by osteoblasts (Takahashi et al., 1988; Abe et al., 1988). This results in osteoclastic bone resorption, causing the release of calcium and phosphate into the extracellular fluid. The ability of $1\alpha,25(OH)_2D_3$, in concert with PTH, to resorb bone is crucial for maintaining calcium and phosphate homeostasis. In addition, $1\alpha,25(OH)_2D_3$ also induces rapid changes in cytosolic calcium levels via nongenomic pathways in osteoblasts (Lieberherr, 1987) and osteosarcoma cells (Caffery and Farach-

Carson, 1989).

Actions on Immune System: $1\alpha,25(OH)_2D_3$ has important physiological actions beyond regulation of calcium-phosphate homeostasis and bone mineralization. The hormone is shown to have noncalcemic actions, such as modulation of cell growth and differentiation of human promyelocytic leukemia (HL-60) cells into monocytes (Abe et al., 1981) and macrophages (McCarthy et al., 1983; Koeffler et al., 1984; Murao et al., 1983). Similar observations in various other cancer cell lines have prompted the development of analogs of $1\alpha,25(OH)_2D_3$ that have minimal hypercalcemic activity but retain the antiproliferative and prodifferentiating properties of the hormone.

Antiproliferative actions of $1\alpha,25(OH)_2D_3$ are exhibited by arresting the cell cycle progression that is associated with upregulation of cyclin-dependent kinases, p21 and p27, and downregulation of cyclins A and D1 ((Liu et al., 1996; Verlinden et al., 1998). In cancer cells, vitamin D compounds have been shown to have inhibitory effects on the mitogenic activity of insulin-like growth factor I and II (Rozen and Pollak, 1999; Nickerson and Huynh, 1999). Other growth factors that are affected by $1\alpha,25(OH)_2D_3$ include vascular endothelial growth factor (Mantell et al., 2000), keratinocyte growth factor (Lyakhovich et al., 2000), epidermal growth factor (Lee et al., 2001), and transforming growth factor-beta (Heberden et al., 1998; Wu et al., 1999). In colorectal cancer, high expression of VDR is associated with good prognosis. Both $1\alpha,25(OH)_2D_3$ and EB1080 (Seocalcitol, a non-hypercalcemic $1\alpha,25(OH)_2D_3$ analog) are shown to induce the expression of E-cadherin (Palmer et al., 2004), a transmembrane protein whose loss of function is associated with colon cancer progression.

Evidence from a number of animal model studies suggests significant beneficial effects of $1\alpha,25(OH)_2D_3$ and its analogs in post-transplant immunosuppression. In combination with cyclosporine or mycophenolate mofetil, immunosuppressive agents in common clinical use for transplantation, $1\alpha,25(OH)_2D_3$ agonists are shown to prolong grafted tissue survival in animal allograft studies, in comparison with either agent used alone (Gregori et al., 2001; Redaelli et al., 2002; Veyron et al., 1993). Findings from such studies provide a basis for testing the use of $1\alpha,25(OH)_2D_3$-related analogs in human transplantation. The primary reason for the delay in clinical applications of vitamin D compounds in the treatment of cancer, organ transplantation, and other immune-mediated diseases is the systemic toxicity of $1\alpha,25(OH)_2D_3$ itself. However, there is now considerable optimism that this hurdle will be overcome with the development of analogs of $1\alpha,25(OH)_2D_3$ that have favorable altered activity.

Apoptosis, or programmed cell death, is a natural regulatory process in the body to maintain tissue homeostasis, but cancer cells fail to undergo apoptosis, resulting in malignant outgrowth (Hansen et al., 2001). It has been shown that $1\alpha,25(OH)_2D_3$ and related analogs induce apoptosis in several cancer cell types by various pathways, including activation of mitogen activated protein (MAP) kinases (Park et al., 2000), upregulation of tumor necrosis factor-alpha (Golovko et al., 2005) and reduction in the expression of bcl-2 (Blutt et al., 2000), a gene associated with inhibition of apoptosis. Direct inhibitory effects of $1\alpha,25(OH)_2D_3$ on antigen- or lectin-stimulated human or murine T-cell activation and proliferation (Bhalla et al., 1984; Rigby et al., 1984; Griffin et al., 2003), IL-2 secretion (Bhalla et al., 1986; Jordan et al., 1989), and cell cycle

progression from early G1 to late G1 phase (Rigby et al., 1985) have also been reported.

Other Actions of 1,25(OH)$_2$D$_3$: 1α,25(OH)$_2$D$_3$ has a wide range of non-classical actions in the body. Receptors for 1α,25(OH)$_2$D$_3$ are present in different non-classical target tissues including muscle, skin, pancreas and brain. In many of these systems, the effect and mode of action of the hormone is unclear.

Muscle cells express VDR, and 1α,25(OH)$_2$D$_3$ has nongenomic actions on skeletal muscle. Evidence of the hormonal stimulation of rapid calcium transport from skeletal muscle to serum in hypocalcemic animals suggests the action of the hormone on muscle may be important for calcium homeostasis of the entire organism (Collins and Norman, 2001). One of the clinical features of profound vitamin D deficiency is severe proximal myopathy and administration of vitamin D has been shown to resolve myopathy within days to weeks.

Experimental evidence of 1α,25(OH)$_2$D$_3$ effects in normal pancreatic secretion of insulin suggests a functional role of 1α,25(OH)$_2$D$_3$ in the endocrine functions of the pancreas (Norman et al., 1980; Cade and Norman, 1986). Experiments in mice have shown that functionally inactive VDR is associated with profoundly reduced insulin secretory capacity and glucose tolerance, independent of alterations in calcium homeostasis (Zeitz et al., 2003). Insufficiency of 1α,25(OH)$_2$D$_3$ is implicated in the pathogenesis of both type 1 (Stene and Joner, 2003; Mathieu et al., 2004) and type 2 (Chiu et al., 2004; Gedik and Akalin, 1986) forms of diabetes.

Skin, renin-angiotensin system in the kidney, and the mammary gland are also considered to be nonclassical targets of 1α,25(OH)$_2$D$_3$ action. In the skin, the hormone

appears to affect cell growth and differentiation. Psoriasis, a chronic hyperproliferative skin disease, is shown to improve on treatment with calcipotriol, a nonhypercalcemic analog of $1\alpha,25(OH)_2D_3$ (Kragballe et al., 1988). Studies have shown that $1\alpha,25(OH)_2D_3$ is a negative regulator of the renin-angiotensin system that plays an important role in regulating blood pressure. Hypertension in VDR-null mice has been shown to be a result of VDR-mediated repression of the renin gene by $1\alpha,25(OH)_2D_3$ (Li et al., 2002). In the mammary gland, the hormone-dependent effects of VDR attenuate mammary development (Zinser et al., 2002).

Vitamin D Deficiency

Deficiency of vitamin D leads to impaired intestinal absorption of calcium, resulting in hypocalcemia. Diminished phosphate clearance associated with renal failure leads to elevated levels of blood phosphate; this change, in turn, further lowers levels of calcium and $1\alpha,25(OH)_2D_3$, stimulating parathyroid hormone excess. The resultant secondary hyperparathyroidism increases the release of calcium and phosphate from bone, ultimately resulting in rickets in children and osteomalacia in adults. Rickets causes progressive softening and weakening of bone structure. Classical skeletal symptoms associated with this disease include knock-knees, bowlegs, curvature of the spine, thoracic deformities, and inadequate mineralization of tooth and enamel (Collins and Norman, 2001). Vitamin D-dependent rickets type I (VDDR I), characterized by hypocalcemia and hyperparathyroidism, is a rare heritable defect of vitamin D activation. Because mutation of 1-hydroxylase gene constitutes the molecular basis of this disorder

(Miller and Portale, 2003), physiologic replacement of 1α-hydroxylated metabolites of vitamin D result in clinical remission. A second rare inherited disorder, vitamin D-dependent rickets type II (VDRR II), also known as hereditary vitamin D-resistant rickets (HVDRR), is characterized by resistance to biologic actions of $1\alpha,25(OH)_2D_3$ (Feldman and Malloy, 1990). Because this disease is associated with hypocalcemia and secondary hyperparathyroidism, it resembles that of vitamin D deficiency, but HVDRR is also accompanied by elevated levels of $1\alpha,25(OH)_2D_3$, distinguishing it from VDDR I. The molecular basis of this disease is mutation of the VDR gene. Although most of the mutations are reported in the DBD of the receptor, mutations in the LBD, including an R274L mutation (Kristjansson et al., 1993), have been reported. The present research focuses, in part, on the functional characterization of R274.

RESEARCH OBJECTIVES

The primary focus of this research is to elucidate the importance of the serine and arginine residues at positions 237 and 274, respectively, present in the LBD of nuclear VDR by studying the interaction between $1\alpha,25(OH)_2D_3$ and mutant forms of the receptor. Three mutant forms of the receptor were constructed using site-directed mutagenesis: S237A, R274A, and a double mutant, S237A/R274A, containing both the mutations. The residues were replaced by alanine to disrupt the hydrogen bonding between these residues and the biologically important 1-hydroxyl group of $1\alpha,25(OH)_2D_3$. Ligand saturation binding assays using $[^3H]$-$1\alpha,25(OH)_2D_3$ were performed to compare how these mutations affect the binding affinity of the receptor in comparison with the wild-type receptor. Western blot analyses using a VDR-specific antibody were done to confirm that all the receptor constructs were expressed as full-length proteins. Partial trypsin digestion experiments using *in vitro* translated receptor (wild-type or mutant) labeled with $[^{35}S]$-methionine were done to study $1\alpha,25(OH)_2D_3$-induced conformational changes of the wild-type and mutated receptors.

MATERIALS AND METHODS

Buffers and Solutions

Buffers

- TE buffer, pH 8.0: 10mM Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCL), 1mM ethylenediaminotetraacetic acid (EDTA).
- TAE buffer: 89 mM Tris, 89 mM acetic acid, 2 mM EDTA.
- TBE buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA.

Bacterial cell culture

- Ampicillin solution (100 mg/mL): Dissolve 1 g ampicillin, sodium salt (Mediatech, Inc.) in 10 mL water. Filter sterilize using 0.2 μm sterile Acrodisc® 13 syringe filter (Gelman Sciences).
- NZY$^+$ Broth: 10 g Bacto Casein Digest (Difco), 1 g Bacto Casamino Acids (Difco), 5 g Yeast Extract (Difco), 5 g Sodium Cholride (Sigma), 1 g Magnesium Sulfate (anhydrous) in 1 L Millipore water. Autoclave at 121°C and 105 kPa chamber pressure for 20 minutes. Store at 4°C.
- LB Broth: 10 g Bacto Tryptone (Difco), 5 g Yeast Extract, 5 g Sodium Chloride in 1 L Millipore water. Autoclave at 121°C and 105 kPa chamber pressure for 20 minutes. Store at 4°C.
- LB Agar selection plates: 10 g Bacto Tryptone, 5 g Yeast Extract, 5 g Sodium Chloride, 15 g Bacto Agar (Difco) in 1 L Millipore water. Autoclave at 121°C

and 105 kPa chamber pressure for 20 minutes. Cool to 55°C and add 1 mL of ampicillin (100 mg/mL) to a final concentration of 100 μg/mL. Mix well and pour approximately 50 mL of the agar solution per plate. Keep overnight at room temperature, then store the plates inverted at 4°C for up to a month.

Eukaryotic Cell Solutions

- Chloroquine solution (8 mM): 0.041 g chloroquin diphosphate (Fluka), 10 mL Millipore water. Filter-sterilize using 2 μm sterile Acrodisc® 13 syringe filter.
- DEAE solution (10 mg/mL): 1.0 g DEAE-dextran hydrochloride per 100 mL phophate buffered saline (10 mM). Filter-sterilize using 0.22 μm polyether sulfone (PES) filter (Corning Inc.).
- Dulbecco's Modification of Eagle's Medium (DMEM): 13.38 g DMEM powder (Cellgro) with 4 mM L-glutamine adjusted to contain 1.5 g sodium bicarbonate and 4.5 g glucose, 90 %; newborn calf serum (NCS) 10 % (BioWhittaker) in 1 L Millipore water. Add 10 mL antibiotic solution (Cellgro 100x: 100,000 U/mL penicillin, 25 μg/mL amphotericin, and 10,000 μg/mL streptomycin). Filter sterilize using 0.22 μm polyether sulfone (PES) filter (Corning Inc.).
- Phosphate Buffered Saline (PBS), pH 7.4 (10 mM): 9.55 g PBS powder (Cellgro) dissolved in 1 L Millipore water. Adjust to pH 7.4, if needed. Autoclave at 121°C and 105 kPa chamber pressure for 20 minutes or filter sterilize using 0.22 μm polyether sulfone (PES) filter (Corning Inc.).
- TED Buffer, pH 7.5: 10 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol (DTT) in 1

L Millipore water. Adjust to pH 7.5.

Western Blot Reagents

- Dilution Buffer, pH 7.2: Tris buffered saline (TBS) (Pierce). 25 mM Tris, 0.15 M sodium chloride in 500 mL Millipore water. Store at 4°C.
- Wash Buffer: Add 5 mL of 10 % Tween®-20 to 1000 mL of dilution buffer. Store at 4°C.
- Blocking Reagent: Add 0.5 mL of 10 % Tween®-20 (Sigma) to 100 mL of StartingBlock™ (TBS) blocking buffer (Pierce). Store at 4°C.
- Chemiluminiscent Substrate Working Solution: Mix equal parts of SuperSignal® West Pico stable peroxide solution (Pierce) and the SuperSignal® West Pico luminol/enhancer solution (Pierce). Stable for 8 hours at room temperature.
- NuPAGE® Transfer Buffer (1X): Add 100 mL methanol to 50 mL NuPAGE® Transfer Buffer (20X) (Invitrogen) and make up the volume to 1 L with Millipore water.

1α,25(OH)$_2$D$_3$

The ligand, 1α,25(OH)$_2$D$_3$, was supplied by Dr. Uskokovich from Roche Pharmaceuticals (Nutley, NJ, USA).

Site-Directed Mutagenesis

Human VDR cDNA cloned into pcDNA1.1 (Invitrogen), an expression vector

containing ampicillin resistant gene (Figure 5), was used as a template to construct desired mutant forms of VDR using site-directed mutagenesis. Two clones of mutant VDRs, S237A and R274A, were produced by introducing point mutations in the cDNA encoding amino acids serine and arginine, to code for alanine, according to the instructions given in the QuikChange® Site-Directed Mutagenesis kit (Stratagene).

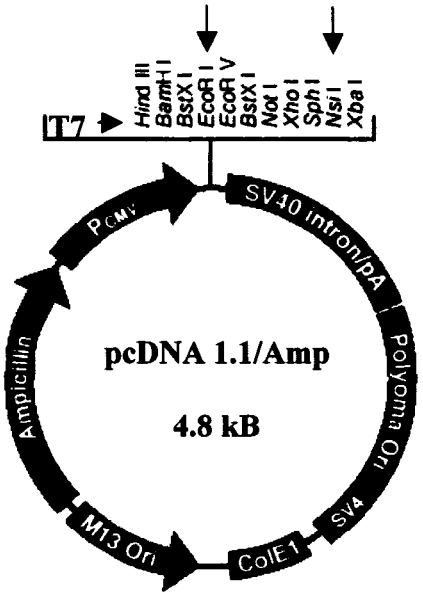


Figure 5. Expression vector pcDNA 1.1. Human VDR cDNA cloned into a pcDNA1.1 (Invitrogen) mammalian expression vector at EcoR I / Nsi I restriction sites, indicated by inverted arrows.

Two complimentary oligonucleotides of 26 bases in length, containing the desired change for each mutant, were obtained from Sigma-Genosys to serve as primers for

generating VDR mutants, S237A and R274A (Table 1). For the double mutant, S237A/R274A, the S237A mutant pcDNA1.1-hVDR expression vector was used as a template with the primer containing mutation for R274A. The reaction consisted of mixing 125 ng of forward primer, 125 ng of reverse primer, 10 ng of pcDNA1.1-hVDR template, 5 μL of 10x reaction buffer, 1 μL of dNTP mix, and nuclease-free water to a final volume of 50 μL. A 1 μL aliquot of of P*fu Turbo* DNA polymerase (2.5 U/ μL) was added. The reaction was placed in MJ Research Minicycler, using a heated lid, and incubated following the program listed below:

"Step1", 95°C for 30 seconds

"Step2" 95°C for 30 seconds

"Step 3" 55°C for 1 minute

"Step 4" 68°C for 12 minutes

Steps 2 through 4 were repeated 16 times.

Following the temperature cycling, the sample reaction was placed on ice for 2 minutes to cool the reaction to $\leq$ 37°C, and then each of the reaction tubes were treated with 1 μL of *Dpn* I restriction enzyme (10 U/mL), mixed gently, and incubated at 37°C for 1 hour to digest the parental (i.e., template) strand.

Table 1. Primers used in the site-directed mutagenesis. (Nucleotides that are underlined and in bold indicate the desired mutations)

Mutant	Primer	Oligonucleotide
S237A	Forward	5'- CCTGGTCAGTTAC**GC**CATCCAAAAGG -3'
	Reverse	5'- CCTTTTGGATG**GC**GTAACTGACCAGG -3'
R274A	Forward	5'- GTCATCATGTTG**GC**CTCCAATGAGTC -3'
	Reverse	5'- GACTCATTGGAG**GC**CAACATGATGAC -3'

Transformation with XL1-Blue Supercompetent Cells

Epicurian coli XL1-Blue supercompetent cells (Stratagene) were gently thawed on ice. For each transformation, an aliquot of 50 μL of supercompetent cells was added to prechilled polypropylene tube (FISHER). Either 1 μL or 4 μL of the *Dpn* I-treated sample was added to separate aliquots of the supercompetent cells. The tubes were swirled to mix the transformation reaction and incubated on ice for 30 minutes. The tubes were then heat shocked for 45 seconds at 42°C in a Precision 183 water bath and then placed on ice for 2 minutes. Next, 0.5 mL of NZY$^+$ broth, preheated to 42°C, was added to each of the transformation reactions and incubated for 1 hour at 37°C with shaking at 225 rpm in a Lab-Line® Orbit Environ-Shaker incubator (Lab-Line Instruments Inc.). Following the 1-hour incubation, the contents of each tube were plated on LB agar plates containing 100 μg/mL ampicillin. The plates were incubated at 37°C for 18 hours in a thermodyne Type 37900 Culture Incubator.

Small-scale Plasmid Purification

Recombinant plasmid was purified from selected transformants using the PerfectPrep® Plasmid Mini kit (Eppendorf), following the protocol suggested by the manufacturer. Single bacterial colonies were used to inoculate 5 mL aliquots of NZY^+ media containing 5 μL of ampicillin (100 mg/ μL). The cultures were incubated for 18 hours at 37°C overnight with shaking at 225 rpm. Next, 3 mL of each bacterial cell culture was pelleted by centrifugation at 13,000 x g for 30 seconds on a Sorval Biofuge Pico Microcentrifuge. The supernatant was aspirated, and the cells were completely resuspended in a 100 μL of resuspension solution by briefly vortexing. The resuspended cells were lysed with 100 μL of lysing solution and mixed several times by gentle inversion of the tubes until the solution became viscous. The bacterial lysate was treated immediately with 100 μL of neutralization solution and thoroughly mixed by inversion to obtain a curd-like granular precipitate. The neutralized lysate was centrifuged at 13,000 x g for 60 seconds to pellet bacterial cell wall debris, precipitated protein and chromosomal DNA. The supernatant containing the plasmid DNA was transferred into a spin column to which 450 μL of DNA binding matrix was added. The spin-column/collection tube assembly was centrifuged at 13,000 x g for 30 seconds, then washed with 400 μL purification solution that contained 70 % ethanol, and centrifuged again for 30 seconds at 13,000 x g. The filtrate was discarded, and the spin column/collection tube assembly was centrifuged again to remove any remaining purification solution. Finally, the DNA was eluted from DNA binding matrix by adding 60 μL of nuclease-free water, pre-heated to 65°C. After brief vortexing and centrifuging

at 13,000 x g for 60 seconds, the eluted DNA was stored at -20°C.

Large-scale Plasmid Isolation and Purification

Once the presence of the desired mutations was confirmed by the dideoxy chain terminator method (described later), clones containing the mutation were propagated to isolate a larger amount of the desired DNA using EndoFree™ Plasmid Maxi purification kit (QIAGEN), following the protocol described by the manufacturer. A single bacterial colony was selected for an agar plate to inoculate 5 mL NZY^+ media containing 5 μL ampicillin (100 μg/mL). The culture was incubated at 37°C for 16 hours with shaking at 250-300 rpm. This starter culture was diluted 1/500 into 250 mL LB-ampicillin medium in a 1000 mL flask. The cell culture was incubated overnight at 37°C with shaking at 250-300 rpm. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C using IEC B-22M centrifuge with an IEC-877 rotor. Supernatant was removed and the pellet was resuspended in a buffer solution (50 mM Tris (hydroxymethyl) aminomethane, pH 8.0; 10 mM ethylenediaminotetraacetic acid (EDTA); 100 μg/mL Rnase A). Next, the cell resuspension was incubated with a lysis buffer solution (200 mM sodium hydroxide, 1 % sodium dodecyl sulfate (SDS) (w/v)) for 5 minutes at room temperature. Neutralization buffer (3.0 M potassium acetate, pH 5.5; pre-chilled to 4°C) was then added to the lysate and mixed gently by inversion. The plasmid DNA was then separated from RNA, proteins, and low molecular weight impurities with a medium-salt wash (1.0 M sodium chloride; 50 mM 3-N-morpholinopropanesulfonic acid (MOPS), pH 7.0; 15 % isopropanyl (v/v)) through

gravity flow on a column provided with the QIAGEN Maxi kit. A high-salt buffer (1.6 M sodium chloride; 50 mM MOPS, pH 7.0; 15 % isopropanyl (v/v)) was used to elute the DNA into a 50 mL Falcon polypropylene tube. Finally, isopropanol was added to the eluted DNA to desalt and precipitate it. The tube was centrifuged for 50 minutes at 7,400 rpm at 4°C and the supernatant was removed. The translucent pellet obtained following centrifugation was washed with 70 % ethanol (70 % ethanol and 30 % endotoxin-free water by volume) at room temperature and separated again by centrifugation for 50 minutes at 4°C at 7,400 rpm. The resulting supernatant was carefully decanted, and the DNA pellet was air-dried and resuspended in 250 μL nuclease-free water. The concentration and purity of the plasmid preparations were determined by UV spectroscopy and agarose gel electrophoresis as described below. All the mutants were also sent to Davis Sequencing Inc. for additional sequencing of the entire VDR cDNA to ensure the absence of any random mutations.

Determination of DNA Concentration

Concentrations of purified plasmids were determined by agarose electrophoresis and by UV spectroscopy. Determination via agarose gel electrophoresis involves prior linearization of the plasmid by *Eco*RI endonuclease digestion. Aliquots of 8 μL of each plasmid preparation were digested with 1 μL of *Eco*RI (20 U/ μL, Promega) in 1x buffer H (Promega). All reaction tubes were incubated at 37°C for 1 hour. Next, 2 μL of blue/orange 6x loading buffer (Promega) was added to each reaction, and the samples were resolved by electrophoresis on a 1 % agarose gel containing 0.005% ethidium

bromide and 1x TAE buffer. The agarose gel electrophoresis was done on a Fisher Scientific FB-SB-710 apparatus at 120 V for 75 minutes. DNA concentration was determined by comparing the intensity of the DNA band to the intensities of the lambda DNA (HindIII/*Eco*RI) markers. A Lambda DNA fragment with equivalent intensity to the DNA band of interest was used to calculate the DNA concentration by the following formula:

$$C_{DNA}\ (\mu g/\mu L) = (N_f/N_{total}) \times C_{Lambda}(\mu g/\mu L) \times V_{DNA} \times F_D$$

where, C_{DNA} is the DNA concentration in μg/μL,

N_f is the size of the matching fragment in base pairs,

N_{total} is the size of the total Lambda DNA, which is 48,02 base pairs,

C_{Lambda} is the concentration of Lambda DNA markers (0.5 μg/μL), and

F_D is the dilution factor, if any.

In addition to gel electrophoresis, DNA concentration and purity were determined by UV absorbance spectroscopy (Ausbel et al., 1997) using a Hewlet Packard 8452A Diode Array spectrophotometer. Suitable dilutions, usually 1:500, of each plasmid DNA preparation were made, and their absorbances were measured at four fixed wavelengths (260 nm and 280 nm). The concentration of the double-stranded DNA was calculated from the following formula:

$$C_{DNA}\ (\mu g/\mu L) = [A_{260} / 0.02\ \mu g/\mu L)] \times F_D$$

where, A_{260} is the absorbance at 260 nm and F_D is the dilution factor. Purity of the DNA

was determined by the ratio of absorbances measured at 260 nm and 280 nm (A_{260} / A_{280}). A ratio of 1.8 to 1.9 is indicative of a highly pure DNA preparation.

DNA Sequencing

DNA sequencing was performed by the dideoxy chain termination method using *fmol*® DNA Cycle Sequencing System (Promega), following the vendor's protocol. Selected pcVDR sequencing primers (Sigma Genosys) were end-labeled (5'-end) with ^{33}P, using Promega's end-labeled primer protocol. The reaction was done using 10 pmol of primer, 10 μCi/μL of [γ-^{33}P]ATP (2,500 Ci/mmol specific activity, Amersham Biosciences), 1 μL of T4 polynucleotide kinase 10x buffer, 1 μL of T4 polynucleotide kinase (10 U/ μL), and nuclease-free water to a final volume of 10 μL. Once the reagents were combined in a 0.5 mL microcentrifuge tube, the tube was incubated at 37°C for 30 minutes, then at 90°C for 2 minutes to inactivate the kinase, and cooled to 4°C using an MJ Research Minicycler. The sequences of all primers that were used for DNA sequencing are listed in Table 2.

Table 2. Primers used for DNA sequencing.

Primer	Sequence
T7	$^{5'}$TAATACGACTCACTATAGGG$^{3'}$
309	$^{5'}$ACTGCCGCATCACCA$^{3'}$
502	$^{5'}$GCGCATCATTGCCAT$^{3'}$
711	$^{5'}$CGTCCAGCTTCTCCA$^{3'}$
935	$^{5'}$CGCTCCAATGAGTCC$^{3'}$
1164	$^{5'}$CGCTGATTGAGGCCA$^{3'}$

For each set of the sequencing reactions, four 0.5 mL microcentrifuge tubes were labeled G, A, T or C. A 2 μL aliquot of the corresponding d/ddNTP mix was added to each tube and kept at 4°C until needed. In a separate microcentrifuge tube, a master mix was prepared for each DNA template by combining 10 ng of the template DNA, 5 μL of *fmol*® sequencing 5x buffer, 1.5 pmol of end-labeled primer, and nuclease-free water to make up a final volume of 16 μL. Finally, 1 μL of sequencing grade *Taq* DNA polymerase (5 U/ μL) was added. After brief mixing and centrifugation, 4 μL of the master mix was added to each of the tubes labeled G, A, T or C, and placed on ice. The reaction tubes were briefly mixed and centrifuged, and quickly placed in a preheated thermal cycler set at 95°C. The cycle sequencing protocol consisted of an initial denaturation step at 95°C for 2min, and 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and an extension at 70°C for 3 minutes (30 seconds per 1000 bp). After the cycle sequencing was completed, 3 μL of *fmol*®

sequencing stop solution was added to each tube to terminate the reaction. The samples were stored at -20°C.

The sequencing reactions were resolved using electrophoresis on a denaturing polyacrylamide gel containing 6.0 M urea. The gel was prepared by mixing 48 mL SequaGel-6 (National Diagnostics), 12 mL SequaGel-6 Buffer (National Diagnostics) and 480 µL of 10 % ammonium persulfate (Sigma). Just prior to loading, the sequencing reactions were heated at 90°C for 2 minutes to denature the DNA, then briefly centrifuged to collect any condensation, and cooled on ice for about a minute. The gel was pre-run before loading the samples (3 µL) at 70 Watts for 45 minutes. The gel was run at 70 Watts for 2 hours on a Sequencing Gel Apparatus Model S2 (Life Technologies, Inc.). The gel was then dried under vacuum (Savant Gel Dryer) at 80°C for 1 hour. Once dried, the gel was placed in an autoradiography cassette with an X-ray BioMax film (Kodak). The film was exposed for 5 days, and then developed on the Kodak RP-X-Omat Model M-7B Processor. The DNA sequence was determined, and the presence of desired mutations and lack of any random mutations was confirmed by BLAST 2 sequence (bl2seq) alignment with the wild-type VDR cDNA sequence, accession number J03258 (National Center for Biological Information, BLAST, Blast 2 Sequences. http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)

Cell Culture

COS-7 Cells

The COS-7 monkey kidney cells (American Type Culture Collection) were

maintained in 150 mm x 25 mm cell culture dishes (Corning Inc.) containing Dulbecco's modification of Eagle medium, DMEM (Cellgro), with 10 % newborn calf serum (Biowhittaker) in a Forma Scientific 37900 incubator at 37°C in a 92 % humidified atmosphere of 95 % air and 5 % CO_2. For preservation, the cells were resuspended in DMEM medium supplemented with 5 % (v/v) dimethyl sulfoxide (DMSO). Aliquots of 1 mL were transferred to prechilled cryovials. The vials were stored at -20°C overnight, then transferred to -70°C for 24 hours, and finally stored in liquid nitrogen. The cells were harvested 48-72 hours after transfection. The media was aspirated from the cell culture dishes, and the cells were washed with 4 mL PBS. Following the removal of buffer, the cells were incubated with 4 mL of 0.25 % trypsin-0.1 % EDTA (Cellgro) at 37°C for 5 to 10 minutes with gentle rocking to ensure all cells were detached from the surface of the culture dish. Trypsin digestion was stopped by the addition of 8 mL DMEM media containing 10 % newborn calf serum. This was transferred into a 50 mL sterile conical tube (Fisher Scientific) and pelleted by centrifugation at ¾ speed for 5 minutes in an IEC HN-S II Centrifuge (International Equipment Company). The supernatant was removed, and the pellet was resuspended in 3 mL of ice-cold TED buffer. The cells were homogenized by extrusion 5 times through a 20-gauge needle. Binding assays were also done with transfected whole cells by resuspending the pellet in 3 mL PBS solution.

FreeStyle™ 293-F cells

FreeStyle™ 293-F cells (Invitrogen) were derived from transformed human

embryonic kidney cells. The cells were maintained in FreeStyle™ 293 Expression Medium in a 250 mL Erlenmeyer Flask (Corning) at 37°C in an incubator (Forma Scientific) with a humidified atmosphere of 8 % CO_2 in air on an orbital shaker platform rotating at 125 rpm. The cap of the flask was loosened a quarter turn from snug to allow oxygenation/aeration. Viability and amount of cell clumping was determined using trypan blue dye exclusion method. A 100 µL aliquot of cell suspension from the shaker flask was transferred to a 1.5 mL microcentrifuge tube containing 400 µL PBS and 500 µL trypan blue dye. The tube was vigorously vortexed for 10-30 seconds to break up cell clumps. A small amount of the mixture was aliquoted to both sides of a hemacytometer. The cells were counted following the hemacytometer instructions. Trypan blue dye stains only dead cells. It is important to count the cells very soon after the dye is added, as living cells lose their capacity to exclude the dye with time. For preservation, the cells were first grown to a desired quantity and harvested when the cell density reached 0.5 to 1 x 10^6 viable cells/mL. The cells were then resuspended in FreeStyle™ 293 Expression Medium containing 10 % DMSO to yield a final density of 5-8 x 10^6 cells/mL. Aliquots of 1 mL were transferred to prechilled cryovials. The vials were stored at -20°C overnight, then transferred to -70°C for 24 hours, and finally stored in liquid nitrogen. For lyphilization of transfected FreeStyle™ 293 cells, 3.2 mL aliquots of lysate were transferred to prechilled cryovials. The vials were immediately transferred to liquid nitrogen and then lyophilized on a LABCONCO Freeze Dry System. After lyophilization, the tubes were stored at -20°C. The cells were harvested 72 hours post-transfection. The transfected cell culture was centrifuged at ½ speed for 5 minutes. The

media was removed by aspiration, and the resulting pellet was washed 3 times with 15 mL of PBS. The final pellet of cells was resuspended in 20 mL TED buffer and homogenized using a 20-gauge needle syringe. A 3 mL aliquot of this was used for each binding assay, and the remaining 17 mL was lyophilized. The lyophilized cell lysate was used for subsequent binding assays by resuspending each vial of cell lysate in 3.2 mL Millipore water.

Transfection

COS-7 Cells

The cells were seeded for transfection at $1.2x10^5$ cells/mL and grown to 70-80 % confluency. After 36 to 48 hours of incubation, the medium was aspirated, and cells were washed with phosphate buffered saline (PBS, pH 8.0), pre-heated in a water bath to 37°C, and allowed to sit for 15 minutes. The wash was aspirated, and the cells were treated with 1.0 mg/mL diethylaminoethyl (DEAE)-dextran (Sigma) in PBS for 9 minutes to facilitate DNA transfection (McCutchan and Pagano, 1968). After exactly 9 minutes, the DEAE-dextran was aspirated, and the cells were washed 2 times with PBS. To each tissue culture dish, 10.6 μg DNA in PBS was added and incubated at 37°C for 30 minutes with gentle rocking every 5 minutes. The construct expression plasmid pcVDR, containing the cDNA for either wild-type of mutant VDR, was used for transfection. Following the 30-minute incubation, DMEM supplemented with 10 % newborn calf serum and 80 μM chloroquin (Sigma) was added to each dish and incubated for 4 hours at 37°C. The medium was then replaced with new DMEM without chloroquine and

allowed to incubate at 37°C for 48-72 hours.

FreeStyle™ 293-F Cells

The cells were seeded for transfection at a cell density of 1 x 10^6 cells/mL in a volume of 30 mL to give a total cell count of 3 x 10^7. On the day of transfection, the suspension culture from the shaker flask was transferred into a sterile disposable, conical centrifuge tube with a screw cap (Fisher Scientific) and centrifuged at ½ speed for 5 minutes in IEC HN-S II Centrifuge at room temperature. The media was aspirated, and the cell pellet was resuspended in approximately half of the original culture volume of fresh, pre-warmed FreeStyle™ 293 Expression Medium by first adding a small volume (10-15 mL) of medium and vortexing for 10-30 seconds to break cell clumps, and then the remainder of the medium was added and mixed well. Viability and total cell count was determined as previously described. Viability of > 90 % was obtained. The volume of the cell culture needed for the experiment was transferred to a sterile shaker flask, and an appropriate volume of fresh, pre-warmed FreeStyle™ 293 Expression Medium was added to obtain a cell density of 1 x 10^6 cells/mL in a 30 mL culture. The shaker flask was placed in a 37°C orbital shaker until the lipid-DNA complexes were prepared for each transfection sample. The lipid-DNA complexes were prepared by diluting 30 μg of plasmid DNA (wild-type or mutant VDR) in Opti-MEM® I to a total volume of 1 mL and by mixing gently. Next, 40 μL of 293fectin™ was diluted in OptiMEM® I to a total volume of 1 mL , mixed gently and incubated for 5 minutes at room temperature. After the 5 minute incubation, the diluted DNA was added to diluted 293fectin™ to obtain a

total volume of 2 mL, mixed gently and incubated for 20-30 minutes at room temperature to allow the DNA-293fectin™ complexes to form. During the DNA-293fectin™ incubation, cell suspension from the incubator was removed, and 28 mL of the suspension was transferred into a sterile 250 mL Erlenmeyer shaker flask. After the completion of DNA-293fectin™ complex incubation, 2 mL of the complex was added to a shaker flask containing 28 mL cell suspension. To the negative control, 2 mL of Opti-MEM® I was added instead of DNA-293fectin™ complex. Each flask contained a total volume of 30 mL with a final density of approximately 1 x 10^6 viable cells/mL. The cells were incubated at 37°C with a humidified atmosphere of 8 % CO_2 in air on an orbital shaker platform rotating at 125 rpm for 72 hours.

Ligand Saturation Binding Assay

Aliquots of 100 µL of crude lysate from COS-7 cells, or FreeStyle™ 293-F cells transfected with cDNA from wild-type or mutant VDR, were incubated for 4 hours at 4°C (or at ambient temperature with transfected COS-7 whole cells) with increasing concentrations (0.125 nM to 6 nM) of [26,27-methyl-^{3}H]-1α,25$(OH)_2D_3$ (specific activity 90 Ci/mmol, Amersham Biosciences) in the presence or absence of a 100-fold excess of unlabelled 1α,25$(OH)_2D_3$. Hydroxyapatite batch assay was used to separate the ligand bound to the receptor from the unbound ligand. Each sample was treated with 300 µL of 50 % hydroxyapatite (BioRad) in TED buffer (Wecksler and Norman, 1979). The hydroxyapatite was washed 3 times by addition of 3 mL TED buffer supplemented with 0.5 % Triton X-100 solution (Sigma) and recovered by centrifugation for 1 minute at

2,245 x g on a swing-bucket rotor in Beckman, Model TJ-6 centrifuge. The final pellet was treated with 1 mL ice-cold 200-proof ethanol to extract the bound ligand. The ethanol containing the ligand was poured into labeled scintillation vials, 3 mL of Ecoscint scintillation liquid (National Diagnostics) was added to each vial, and the bound ligand was quantified on a Beckman LS 6500 scintillation counter.

Western Blot Analysis

Serial dilutions of 1:1, 1:2, and 1:4 of FreeStyle™ 293-F cell homogenate samples of 72 hours post-transfection and untransfected cells were prepared. To 7.5 µL of cytosol, a 2.5 µL aliquot of 4X NuPAGE® LDS Sample Buffer (Invitrogen) was added. The samples were heated for 10 minutes at 70°C and then resolved on 4-12 % NuPAGE® Bis-Tris gels in the Xcell SureLock™ Mini-Cell at 200 V constant voltage for 35 minutes. Following electrophoresis, the proteins were transferred from the gel to a nitrocellulose membrane (Pierce) in a Xcell II™ Blot Module with 1X transfer buffer containing 10 % methanol at 30 Volts. After the transfer, the gel was stained using SimplyBlue™ SafeStain microwave protocol (Invitrogen) to verify protein transfer to the membrane. The membrane was incubated with blocking reagent for 1 hour with shaking to block nonspecific sites. Next, the membrane was incubated with rat anti-hVDR (USBiological) at 1:2000 in blocking reagent for 1 hour with shaking at room temperature or incubated overnight at 2-8°C. The membrane was washed 5 times in wash buffer for 5 minutes. Following the wash step, the membrane was incubated with ImmunoPure® goat anti-rat IgG (H+L) horseradish peroxidase (HRP) conjugate (Pierce)

and diluted 1:20,000 in blocking reagent for 1 hour at room temperature with shaking. The wash step was repeated to remove non-bound HRP-conjugate. Finally, the membrane was incubated for 5 minutes with SuperSignal® West Pico chemiluminescent substrate working solution. Excess reagent was drained, and the membrane was exposed for 15 minutes to detect protein by chemiluminescence.

Transcription and Translation of hVDR for Protease Sensitivity Assay

The hVDR was transcribed and translated *in vitro* using the TnT® Coupled Transcription/Translation System (Promega). An aliquot of 40 μL of TnT® master mix was combined in a 0.5 mL microcentrifuge tube with 1 μg of plasmid DNA (wild-type or mutant VDR), 2 μL of [^{35}S]-methionine (specific activity x Ci/mmole), and nuclease-free water to make up the total volume to 50 μL. The reaction was incubated for 90 minutes at 30°C.

Protease Sensitivity Assay

A 5 μL aliquot of [^{35}S]-hVDR was incubated with 0.5 μL of 10^{-8} M 1α,25(OH)$_2$D$_3$ or ethanol for 10 minutes at room temperature prior to exposure to 0.5 μL of varying concentrations of trypsin (0, 5, 10, 15, 20, 25, 35, 50 and 100 ng/ μL) for 10 minutes. The reaction was stopped by heating at 70°C for 10 minutes in 6 μL of 4 x NuPAGE® lithium dodecyl sulfate (LDS) sample buffer, and then resolved on a 4-12% NuPAGE® Bis-Tris gel (NuPAGE® Electrophoresis System, Invitrogen), dried and autoradiographed. The hormone-dependent sensitivity of wild-type and mutant VDRs,

237A, R274A, and S237A/R274A, to limited proteolysis using a 20 ng/μL concentration of the protease was also determined as described above. Different concentrations of the hormone ranged from 10^{-12} M to 10^{-8} M.

RESULTS

Construction and Verification of Desired Mutant Forms of VDR

The focus of this research is to elucidate the importance of the amino acid residues S237 and R274 present in the LBD of VDR using mutational analysis. Both S237 and R274 are known to interact directly with 1-hyroxyl group of $1\alpha,25(OH)_2D_3$ (Rochel et al., 2000). Point mutations were introduced in the cDNA encoding VDR to disrupt hydrogen bonding between these residues and the hormone. The three mutant forms of the receptor that were constructed are VDR-S237A, VDR-R274A, and a double mutant. VDR-S237A/R274A, containing both the mutations. For the plasmid encoding VDR variant S237A, two point mutations were introduced to change the AGC codon for serine to the GCC codon for alanine at residue 237. For the second mutant, VDR-R274A, the CGC codon for arginine was changed to the GCC codon for alanine at position 274. For the third mutant, VDR-S237A/R274A, codons for serine and arginine at positions 237 and 274, respectively, were both changed to codons for alanine. The plasmids containing altered cDNAs were used to transform competent *E.coli* cells. Colonies were selected for DNA sequencing to verify the presence of desired mutations. The sequences in Figures 6A and 6B confirm the mutations of residues 237 and 274 to alanines. The complete nucleotide sequences for wild-type and variant VDRs are shown in appendices C and D, respectively.

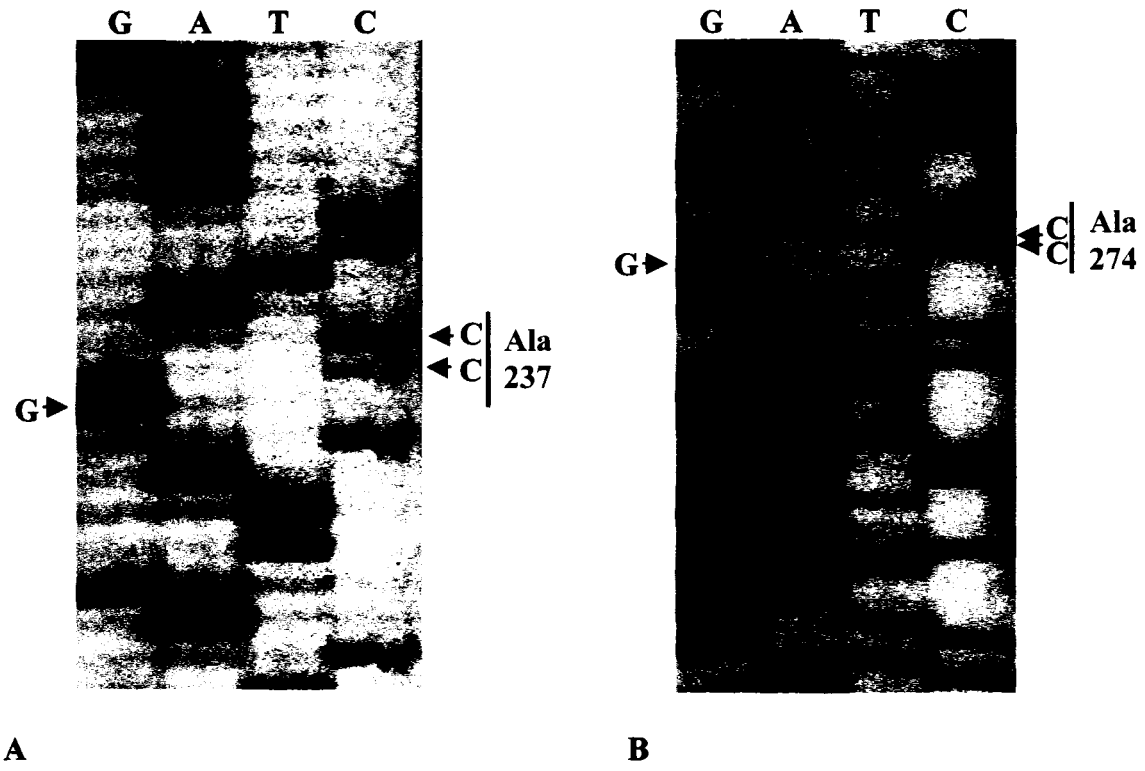


Figure 6. Sequence analysis of the plasmids encoding cDNA of mutant VDR-S237A and VDR-R274A. Plasmid pcDNA1.1 that contained the coding sequence for VDR mutant was purified from bacterial culture using Plasmid Maxi Prep (QIAGEN) and sequenced by the dideoxy chain terminator method. For plasmid encoding cDNA of S237A **(A)**, the indicated GCC codon represents a serine to alanine mutation at residue 237, and for plasmid encoding cDNA of R274A **(B)**, the indicated GCC codon represents an arginine to alanine mutation at 274.

Ligand Saturation Binding Assays

Ligand saturation binding assays using [^{3}H]-1α,25(OH)$_2$D$_3$ were done to compare the relative binding affinities of wild-type and mutant forms of VDR for the hormone. At least three binding assays were done for each receptor construct. Raw data was obtained as disintegrations per minute (DPM) and the graphs for each of the experiments are shown in appendices E and G, respectively. For each experiment, total binding was determined in triplicate by incubating either FreeStyle™ 293-F cell lysate or COS-7 cell lysate that expressed either wild-type or a VDR variant, as a function of increasing concentrations of [^{3}H]-1α,25(OH)$_2$D$_3$. Nonspecific binding was determined in duplicate by incubation with [^{3}H]-1α,25(OH)$_2$D$_3$ along with a 100-fold excess of radio-inert 1α,25(OH)$_2$D$_3$. The difference between the resulting averages for total binding and nonspecific binding yields the specific binding, which is the quantity of receptor that is bound to 1α,25(OH)$_2$D$_3$. DPM values were converted to units of femtomoles using the following equation:

DPM counts / (2.22 x 10^{12} DPM/Ci) = Ci of [^{3}H]-1α,25(OH)$_2$D$_3$.

Ci [^{3}H]- 1α,25(OH)$_2$D$_3$ is then divided by the specific activity of [^{3}H]- 1α,25(OH)$_2$D$_3$, expressed in Ci/mmol, to give mmols of [^{3}H]- 1α,25(OH)$_2$D$_3$.

Femtomoles of receptor was graphed as a function of nanomolar concentrations of [^{3}H]-1α,25(OH)$_2$D$_3$ using nonlinear regression (GraphPad Prism software, version 3.2). The one-site binding dissociation constant (K_D) and BMAX were calculated. The

dissociation constant, K_D, expressed in nanomoles/liter, is the concentration of the ligand required to occupy half of the receptors at equilibrium. The BMAX value represents the total number of receptors present.

Binding data obtained using the COS-7 cells was variable and gave very high nonspecific binding (Table 3 and Appendix F). The K_D values obtained for each receptor construct from VDR-deficient COS-7 cells at ambient temperature (using whole cells) and 4°C (using lysed cells) are summarized in Table 3. In addition to high nonspecific binding, the assay results were not reproducible, and, in some experiments, the K_D values were not significantly different from that of the wild-type receptor. In order to overcome this, the experiments were repeated with FreeStyle™ 293-F cells, a high expression cell line. It was found that nonspecific binding was significantly reduced with these cells. To compare the efficiency of transfection between FreeStyle™ 293-F cells and COS-7 cells, raw data obtained in DPM using the highest concentration of [^{3}H]-1α,25$(OH)_2D_3$ is shown in Table 4. The saturation binding curve shown in Figure 7 represents one of the saturation binding assays with FreeStyle™ 293-F cells that expressed the wild-type VDR. The S237A mutant VDR bound [^{3}H]-1α,25$(OH)_2D_3$ with a K_D of 8.06 ± 3.09 nM, an approximately 4-fold lower affinity than the wild-type VDR K_D of 2.36 ± 0.61 nM, assayed at the same time (Table 5). Binding was undetectable with the R274A mutant VDR and the VDR double mutant. The raw data in disintegrations per minute for the VDR-R274A mutant and VDR-R274A/S237A mutant are similar to that obtained with untransfected cells (Table 6).

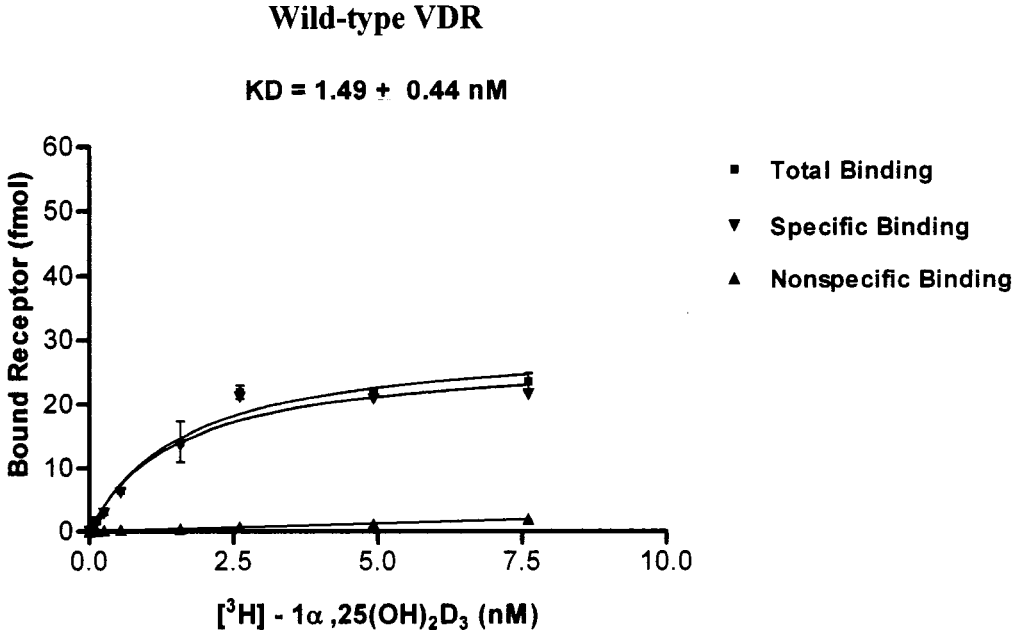


Figure 7. Ligand saturation binding assay. Aliquots of 100 μL cytosol that expressed either wild-type or mutant VDR were incubated at 4°C for 4 hours with increasing concentrations of 1,25-dihydro-[26,27-methyl-^{3}H]-vitamin D_3 (specific activity 90 Ci/mmole) in the presence or absence of 100-fold nonradioactive $1\alpha,25(OH)_2D_3$. Hormone-bound receptor was separated from free ligand using the hydroxyapatite batch assay. Specific binding was calculated by subtracting nonspecific binding from total binding. The data for total binding are the mean of triplicate points, and that for nonspecific binding are the mean of duplicate points. Error bars on the total binding curve are calculated based on the standard deviation of the triplicate data points.

Table 3. Summary of ligand saturation binding assay results with COS-7 cells.

VDR Construct	K_D (nM)	Temperature
Wild-type	3.74 ± 2.33	4°C
	0.79 ± 0.34	Room temperature
	0.33 ± 0.19	Room temperature
S237A	1.24 ± 0.99	4°C
	0.33 ± 0.14	Room temperature
R274A	No Binding	4°C
	0.19 ± 0.02	Room temperature
	0.10 ± 0.04	Room temperature
	0.42 ± 0.22	Room temperature
S237A/R274A	3.90 ± 3.18	4°C
	0.21 ± 0.12	Room temperature
	0.59 ± 0.12	Room temperature

Table 4. Comparison of raw binding data in DPM obtained with COS-7 cells and FreeStyle™ 293-F cells that expressed wild-type VDR with 6 nM [^{3}H]-1,25(OH)$_2$D$_3$.

Cell Type	Total Binding			Nonspecific Binding	
COS-7	393.35	445.90	402.56	278.22	283.20
FreeStyle™ 293-F cells	6406.30	5297.34	5361.29	460.98	501.42

Table 5. Summary of ligand saturation binding assay results with FreeStyle™ 293-F cells.

VDR Construct	K_D (nM)
Wild-type	2.36 ± 0.61 (n = 5)
S237A	8.06 ± 3.09 (n = 4)*
R274A	Undetectable binding (n = 3)
S237A/R274A	Undetectable binding (n = 3)

Data are expressed as means and standard deviation of mean for "n" independent experiments performed at room temperature. Unpaired t test was performed using Graphpad Prism version 3.2, Graphpad Software, CA.
*Significantly different than wild-type VDR, $p<0.004$.

Table 6. Raw binding data in DPM with FreeStyle™ 293-F cells using 6nM [^{3}H]-1α,25(OH)$_2$D$_3$.

VDR Construct	Total Binding			Nonspecific Binding	
Wild-type	6406.30	5297.34	5361.29	460.98	501.42
S237A	2216.75	1732.73	1934.39	438.29	328.25
R274A	476.86	460.28	483.47	368.40	316.83
S237A/R274A	308.68	305.40	368.62	617.59	283.21
Untransfected cells	494.11	383.32	343.57	335.46	365.42

Western Blot Analysis

Since the VDR mutant R274A and the double mutant S237A/R274A showed undetectable binding with [^{3}H]-1α,25(OH)$_2$D$_3$, Western blot analysis was performed to confirm the expression of the variant and wild-type VDRs by the FreeStyle™ 293-F cells. A negative control with untransfected FreeStyle™ 293-F cells was also performed to verify if the cells expressed endogenous VDR. Serial dilutions of 1:1, 1:2 and 1:4 of 72 hours post-transfection cell homogenate samples were resolved on 4-12 % NuPAGE® Bis-Tris gel and electroblotted on nitrocellulose membrane. The membrane was incubated with a monoclonal primary antibody, rat anti-hVDR, followed by a wash step, and then incubated with a secondary antibody, goat anti-rat IgG, horseradish peroxidase conjugate. After washing off the unbound secondary antibody, a chemiluminescent substrate was used to detect the monoclonal antibody interaction with VDR. As shown in

Figure 8, the monoclonal antibody interacted with a protein of molecular weight 49 kDa that corresponds to VDR. The negative control with untransfected cells did not show any bands, indicating that the cells do not express endogenous VDR.

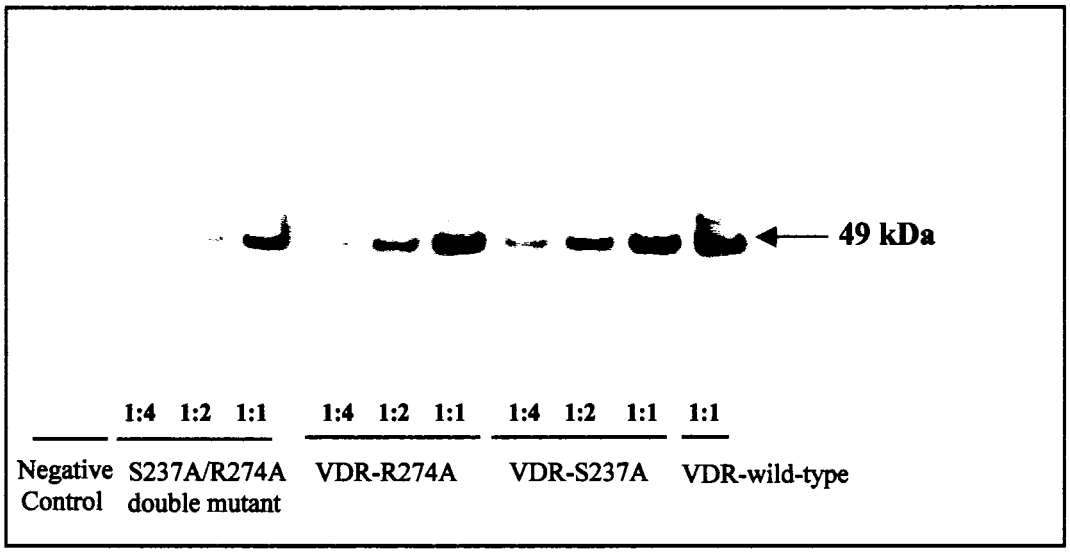


Figure 8. Western blot analysis. Serial dilutions of 1:1, 1:2 and 1:4 of 72 hours post-transfection and untransfected cell homogenate samples were resolved on 4-12 % gradient polyacrylamide gel and electroblotted on nitrocellulose membrane. The membrane was incubated with blocking reagent to block nonspecific sites. Next, the membrane was incubated with a 1:2000 dilution of primary antibody, rat anti-hVDR, followed by a wash step, and then incubated with a 1:20,000 dilution of secondary antibody, goat anti-rat IgG and horseradish peroxidase conjugate. After washing off the unbound secondary antibody, a chemiluminescent substrate was used to detect the monoclonal antibody interaction with VDR.

Protease Sensitivity Assay

Limited proteolytic digestion experiments have previously been used as a tool to analyze conformational changes within proteins (Allan et al., 1992; Peleg et al., 1995; Väisänen et al., 1998). In the present study, *in vitro* translated VDR labeled with [^{35}S]-methionine was digested for a limited time using increasing concentrations of trypsin, either in the presence or absence of saturating concentrations (10^{-8} M) of 1α,25(OH)$_2$D$_3$. In the absence of trypsin or hormone, the 46 kDa band corresponds to the VDR (Figure 9). In the absence of the hormone, trypsin digestion for a limited time (10 minutes) resulted in degradation of the protein. However, under the same incubation conditions, in the presence of hormone, proteolytic resistant fragments of molecular masses 30 kDa and 20 kDa were observed for digestion with trypsin. With increasing concentrations of trypsin, the intensity of 30 kDa band decreased, indicating further proteolysis of the ligand-protected fragment. The band intensity of the 20.1 kDa digestion product increased as the 30 kDa band was degraded.

Trypsin sensitivity assays have also been done with *in vitro* translated [^{35}S]-VDR variants to determine if the mutations induce any unique pattern of sensitivity to trypsin. Figures 10-13 show the hormone-dependent sensitivity of wild-type and mutant VDRs, S237A, R274A, and S237A/R274A, respectively, to limited proteolysis using a 20 ng/μL concentration of the protease. The hormone concentrations ranged from 10^{-12} M to 10^{-8} M. For the mutant VDRs S237A (Figure 11) and R274A (Figure 12), trypsin digestion products corresponding to molecular weights of 30 kDa and 20.1 kDa were observed, albeit at a lower band intensity as compared to that of the wild-type receptor (Figure 10).

These bands were observed for the mutant receptors both in the absence and presence of the hormone. For the S237A/R274A double mutant (Figure 13), the 30 kDa fragment was only barely visible at higher concentrations of the $1\alpha,25(OH)_2D_3$ and not seen at all with lower concentrations of the ligand.

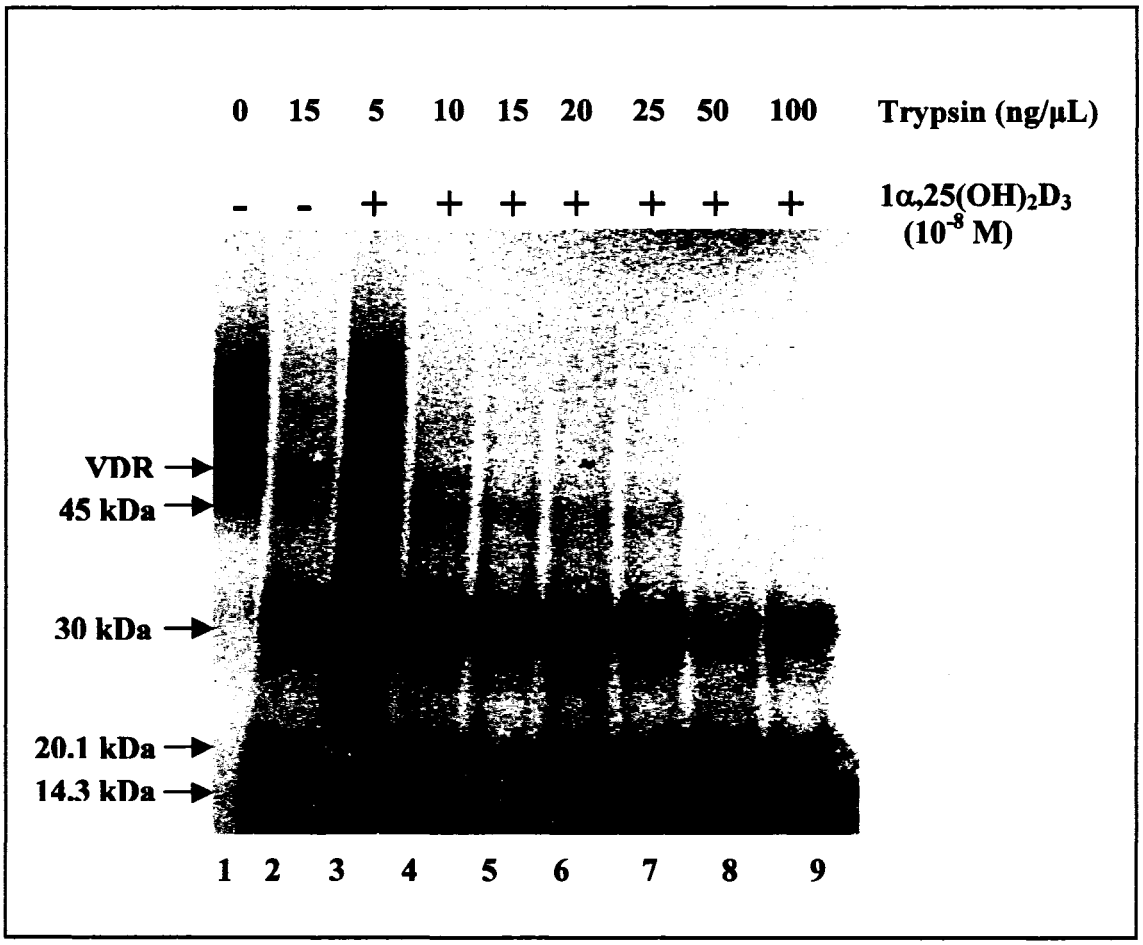


Figure 9. 1α,25(OH)$_2$D$_3$-dependent sensitivity of wild-type VDR. An aliquot (0.5 μL) of *in vitro* translated VDR labeled with [^{35}S]-methionine was preincubated at room temperature with 10^{-8} M 1,25(OH)$_2$D$_3$ (lanes 3-9) or 0.1% ethanol (lanes 1 and 2) for 10 minutes before exposure them to partial proteolytic digestion with increasing concentrations of trypsin. The digestion products were heated in 1X LDS loading buffer for 10 minutes at 65°C, electrophoresed on a 4-12% gradient polyacrylamide gel, dried, and then detected by autoradiography.

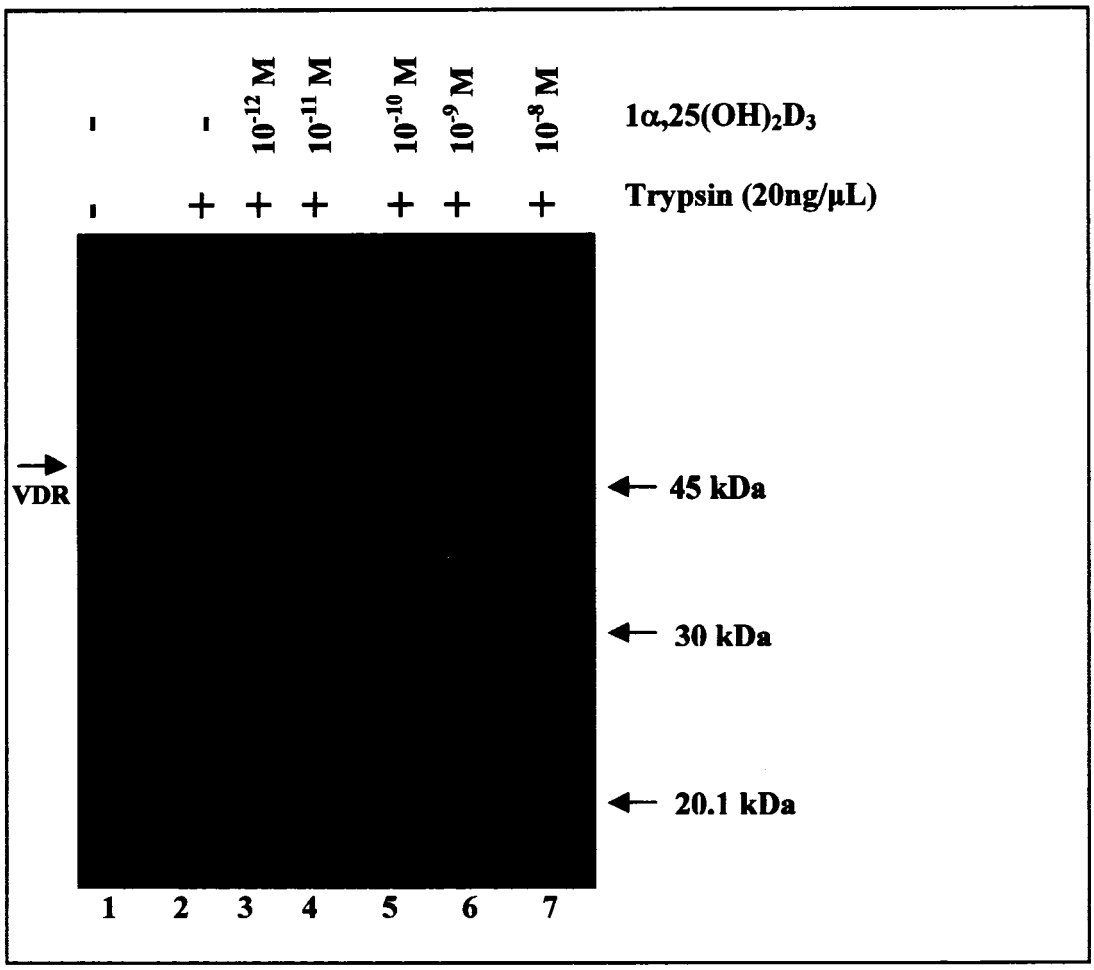


Figure 10. Protease sensitivity studies of hVDR. Sensitivity of [^{35}S]-methionine labeled, *in vitro* translated, wild-type VDR to limited proteolysis as a function of ligand concentration. An aliquot (0.5 μL) of *in vitro* translated wild-type VDR was preincubated for 10 minutes at room temperature with the indicated concentrations of 1α,25(OH)$_2$D$_3$ (lanes 3-7) or ethanol (lanes 1 and 2), then digested with 20 ng/μL of trypsin for 10 minutes. The digestion products were heated in 1X LDS loading buffer for 10 minutes at 65°C, separated by electrophoresis on a 4-12% gradient polyacrylamide gel, dried, and then detected by autoradiography.

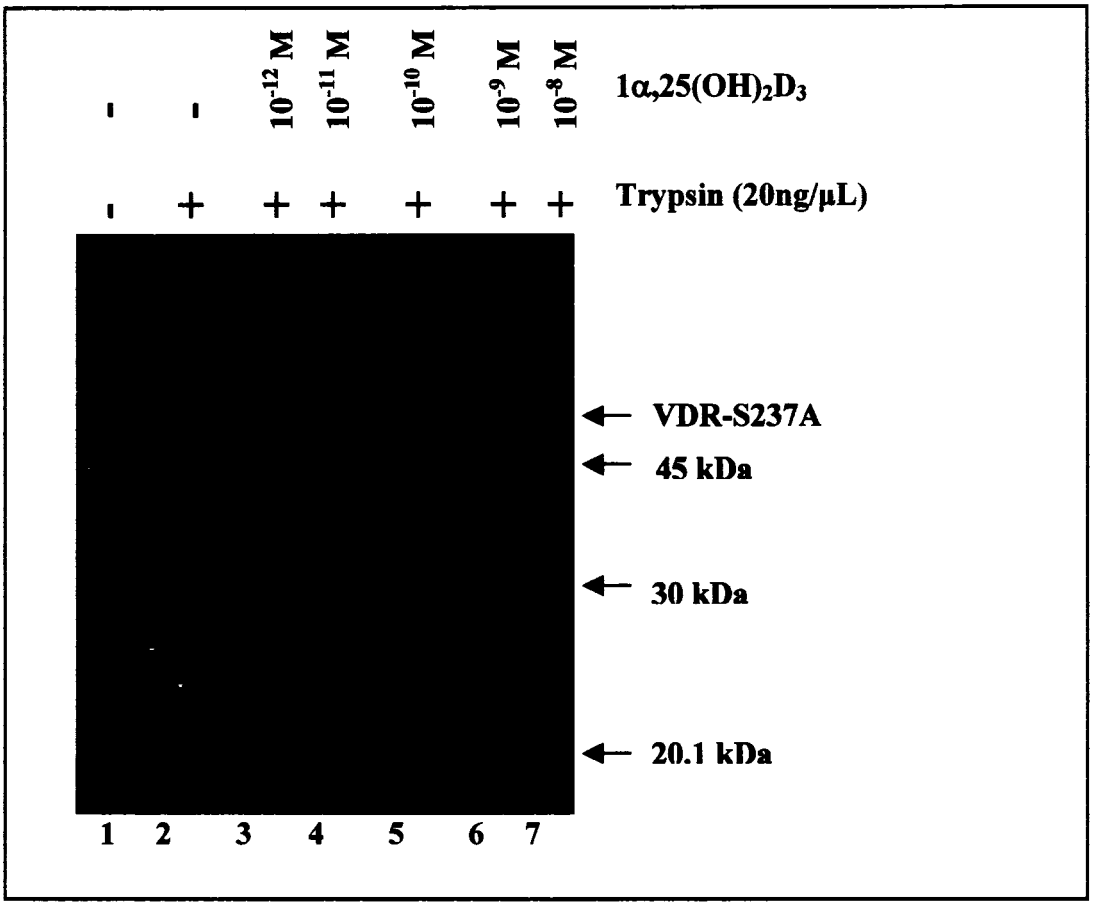


Figure 11. Protease sensitivity studies of VDR-S237A mutant. Sensitivity of [^{35}S]-methionine labeled, *in vitro* translated, VDR-S237A to limited proteolysis as a function of ligand concentration. An aliquot (0.5 μL) of *in vitro* translated VDR-S237A was preincubated for 10 minutes at room temperature with the indicated concentrations of 1α,25(OH)$_2$D$_3$ (lanes 3-7) or ethanol (lanes 1 and 2), then digested with 20 ng/μL of trypsin for 10 minutes. The digestion products were heated in 1X LDS loading buffer for 10 minutes at 65°C, separated by electrophoresis on a 4-12% gradient polyacrylamide gel, dried, and then detected by autoradiography.

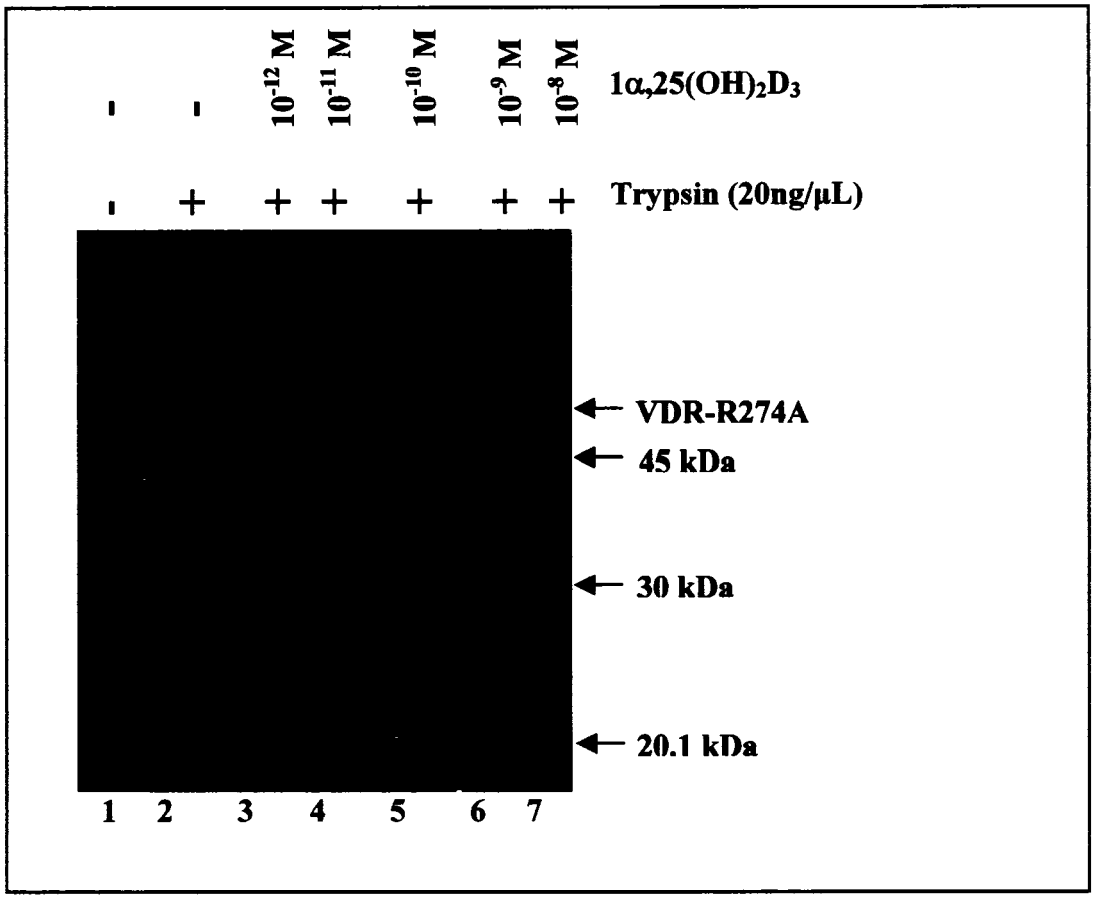


Figure 12. Protease sensitivity studies of VDR-S274A mutant. Sensitivity of [^{35}S]-methionine labeled, *in vitro* translated, VDR-R274A to limited proteolysis as a function of ligand concentration. An aliquot (0.5 μL) of *in vitro* translated VDR-R274A was preincubated for 10 minutes at room temperature with indicated concentrations of 1α,25(OH)$_2$D$_3$ (lanes 3-7) or ethanol (lanes 1 and 2), then digested with 20 ng/μL of trypsin for 10 minutes. The digestion products were heated in 1X LDS loading buffer for 10 minutes at 65°C, separated by electrophoresis on a 4-12% gradient polyacrylamide gel, dried, and then detected by autoradiography.

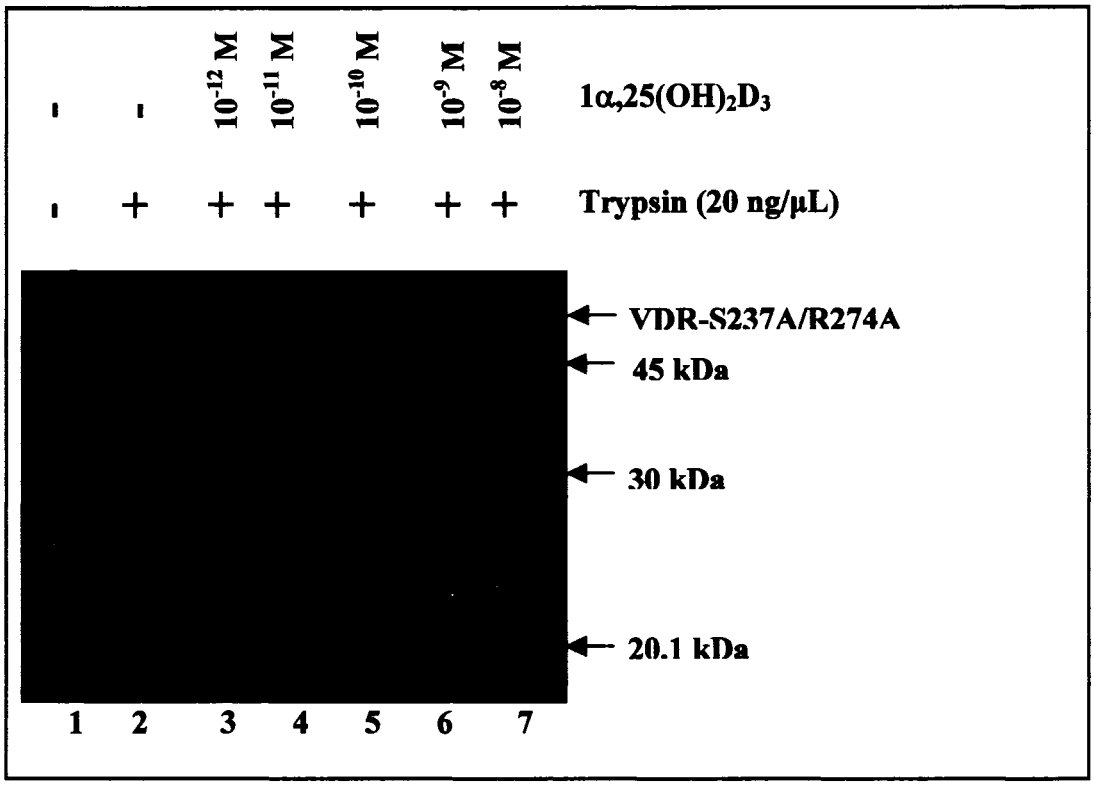


Figure 13. Protease sensitivity studies of VDR-S237A/R274A double mutant. Sensitivity of [^{35}S]-methionine labeled, *in vitro* translated, VDR-S237A/R274A to limited proteolysis as a function of ligand concentration. An aliquot (0.5 μL) of *in vitro* translatedVDR-S237A/R274A double mutant was preincubated for 10 minutes at room temperature with indicated concentrations of 1α,25(OH)$_2$D$_3$ (lanes 3-7) or ethanol (lanes 1 and 2), then digested with 20 ng/μL of trypsin for 10 minutes. The digestion products were heated in 1X LDS loading buffer for 10 minutes at 65°C, separated by electrophoresis on a 4-12% gradient polyacrylamide gel, dried, and then detected by autoradiography.

DISCUSSION

Binding of $1\alpha,25(OH)_2D_3$ to its cognate receptor is an essential, early step in the signal transduction pathway that triggers a cascade of events, leading to regulation of hormone sensitive gene expression. Understanding the various biological actions of $1\alpha,25(OH)_2D_3$ requires knowledge of not only how the hormone interacts with its cognate receptor, but also how subtle conformational changes in the receptor upon ligand binding can affect the interaction of the receptor-ligand complex with coactivators and basal transcriptional machinery. These interactions may result in changes in gene transcription and, therefore, protein expression.

The present study focuses on the interactions between specific mutated forms of the VDR and $1\alpha,25(OH)_2D_3$ by comparison with wild-type receptor. Mutational analysis has been used to elucidate the importance of residues S237 and R274 present in helices 3 and 5, respectively, in the steroid binding region of the receptor. The first natural mutation found in the LBD of the VDR was a missense mutation (CGC → CTC) that resulted in substitution of a positively charged arginine residue at position 274 with a neutral leucine. This mutation was identified in a severely debilitated patient with HVDRR (Kristjansson et al., 1993). From the crystallographic studies of the deletion mutant (Δ 165-215) of the VDR ligand binding domain complexed with $1\alpha,25(OH)_2D_3$, it is known that residues S237 and R274 form hydrogen bonding interactions with the biologically important 1-hydroxyl group of $1\alpha,25(OH)_2D_3$. In addition to forming hydrogen bonds with the hormone, the R274 residue in helix 5 is also hydrogen bonded

to water molecules to form an open water channel between loop1-3 and helix 3 (Rochel et al., 2000). To abolish the hydrogen bonding interactions, these polar residues have been replaced with alanine by introducing mutations in the wild-type VDR cDNA using site-directed mutagenesis. The three mutant forms of the receptor that were created are VDR-S237A, VDR-R274A and a double mutant, VDR-S237A/R274A, containing both of the mutations. The cDNA of wild-type or mutant VDRs was transfected into COS-7 cells or FreeStyle™ 293-F cells to evaluate the relative binding affinities of the wild-type and variant VDRs. Western blot analysis was done using VDR-specific antibody to confirm the expression of the wild-type and variant VDRs by FreeStyle™ 293-F cells. In order to assess the ability of the $1\alpha,25(OH)_2D_3$ to induce conformational changes in VDR upon ligand binding, the ligand-VDR complexes were subjected to limited proteolysis with trypsin. The hormone-induced conformational changes of the mutant receptors were also studied by partial trypsin digestion experiments.

Ligand Saturation Binding Analysis

Relative binding affinities of mutated and wild-type receptors were determined by $[^3H]$-$1\alpha,25(OH)_2D_3$ binding assays. When the experiments were done with VDR-deficient COS-7 cells, nonspecific binding was high, and the data showed that there was no significant difference between the binding affinity of wild-type VDR and VDR variants (Table 3). When the hydrogen bonding interactions between the 1-hydroxyl group of the hormone and residues S237 and R274 are disrupted by replacing the polar residues at these positions with alanines, the binding affinity of the mutated receptor for

the hormone, especially that of the double mutant, would be expected to be significantly affected. However, the K_D values for mutant receptors were very similar to the K_D values obtained with the wild-type VDR. Besides being counter-intuitive, these results did not agree with previously published data (Kristjannson et al., 1993; Väisänen et al., 1998; Väisänen et al., 1999; Yamamoto et al., 2000; Choi et al., 2001; Gardezi et al., 2001; Kittaka et al., 2003), which led to the discovery that low levels of endogenous VDR were being expressed. Therefore, the experiments were repeated using VDR deficient, high expression FreeStyle™ 293-F cells. The binding data obtained with these cells were found to have much better signal-to-noise ratio than those obtained with COS-7 cells. Table 4 illustrates a comparison of raw ligand saturation binding data obtained in DPM using COS-7 and FreeStyle™ 293-F cells.

The K_D value for the wild- type VDR is 2.36 ± 0.61 nM and falls well within the range of the previously reported K_D values, which are between 1-50 X 10^{-10} M (Wecksler and Norman, 1980; Wecksler et al., 1980a; Wecksler et al., 1980b). The binding results suggest that S237 plays an assistant role, whereas R274 is critical for proper anchoring of the hormone within the ligand binding pocket to form hydrogen bonds with the 1-hydroxyl group of the ligand. Mutant S237A with a K_D value 8.06 ± 3.09 nM, exhibited an approximately 4-fold lower affinity for $1\alpha,25(OH)_2D_3$ than the wild-type VDR, with a K_D value of 2.36 ± 0.61 nM, when assayed at 4°C using a lysate of transfected FreeStyle™ 293-F cells. With the R274A mutant, binding with $[^3H]$-$1\alpha,25(OH)_2D_3$ was undetectable. These findings indicate residue S237 to be important but not essential for binding, unlike R274, which is absolutely essential for hormone binding. As expected,

the ability of the double mutant receptor, S237A/R274A, to bind the ligand was completely abolished. Western blot analysis of the wild-type and VDR variants was performed using the rat anti-hVDR antibody. The analysis revealed full-length expression of all the receptor proteins by FreeStyle™ 293-F cells.

Mutant VDR R274A is similar to the HVDRR mutant, R274L, in that both mutations result in the loss of the ability to form hydrogen bonds with the 1-hydroxyl group of the $1\alpha,25(OH)_2D_3$. However, in the R274A mutant, in addition to removing the hydrogen bond, alanine substitution also introduces a space due to alanine's smaller size in comparison with the size of arginine. Kristjansson et al., have previously shown a lack of activity in ligand binding and transactivation for the R274L VDR mutant, indicating that R274 plays a crucial role in proper binding of the hormone within the ligand binding cavity (Kristjansson et al., 1993). Yamamoto et al., have shown that the ability of the VDR-R274A mutant to bind the natural ligand was undetectable, and that of VDR mutant S237A was significantly decreased by approximately 5-fold (Yamamoto et al., 2000), which is in agreement with the results obtained from the present study. Alanine scanning mutational analysis of the residues lining the ligand binding cavity by Choi et al., indicate that the amino acid residues R274 and S237 are important for transcriptional activation (Choi et al., 2001; Choi et al., 2003). Transactivational activity of various vitamin D ligands in conjunction with alanine scanning mutations of the residues lining the ligand binding pocket was evaluated (Choi et al., 2003). Their work revealed that the mutation S237A has a moderate effect (20 % - 60 % of the original activity of wild-type VDR) on $1\alpha,25(OH)_2D_3$, and little effect (61 % - 90 %) on the potency of the 20-epivitamin D

analogs, 20-epi-1,25$(OH)_2D_3$ and 22-methyl-20-epi-1,25$(OH)_2D_3$. The transcriptional potency of S237A mutant was increased with the 20-epi ligands, KH1060 and 22-ethyl-20-epi-1,25$(OH)_2D_3$. These results indicate that mutating the S237 residue to a less bulky alanine residue tends to increase the potency of the highly active 20-epi-ligands. The R274 residue was found to be essential for transactivation by the natural ligand, 1α,25$(OH)_2D_3$, as well as the analogs (Choi et al., 2003). In 2001, Gardezi et al., showed that the analog 1β-hydroxymethyl-3-epi-16-ene-26a,27a-bishomo-25D_3 (JK-1626-2), which has two modifications on the A-ring (1β-hydroxymethyl and 3α-hydroxyl groups) was able to restore the transcriptional potency of the HVDRR mutant R274L more effectively than the natural hormone despite having a transcriptional potency very similar to 1α,25$(OH)_2D_3$ in the wild-type VDR. They have also shown that the greater potency of the analog to transactivate the R274L mutant VDR was because of better receptor binding, as determined by quantitative protease sensitivity assays. Their experiments showed that the R274L mutation diminished the ability of 1α,25$(OH)_2D_3$ to stabilize the receptor conformation such that only weak 1α,25$(OH)_2D_3$-mediated resistance to trypsin was detected at 1 μL. In contrast, the JK-1626-2 analog maintained a significant ability to stabilize receptor conformation at 100 nM, suggesting that the analog has at least 100-fold greater binding affinity than the natural hormone. The binding results from the present study, taken together with previously published results (Kristjansson et al., 1993; Choi et al., 2001; Choi et al., 2003; Yamamoto et al., 2000; Gardezi et al., 2001) confirm the importance of the 1α-OH group of the steroid hormone for binding to the VDR and the importance of amino acid residues S237 and especially R274 in this binding.

Protease Sensitivity Analysis

Proteolytic analysis has been used as a technique to analyze the ligand binding induced conformational changes of proteins (Allan et al., 1992). Other research groups have used a similar approach to analyze conformational changes in VDR upon ligand binding (Peleg et al., 1995; Väisänen et al., 1998). Since the [^{3}H]-1α,25(OH)$_2$D$_3$ binding activity of mutants such as the HVDRR mutant, R274L, is poor, competition assays with unlabeled vitamin D analogs cannot be used to assess their relative affinity for the mutant. Also, radiolabeled analogs are not commercially available for direct binding analysis. In order to circumvent this problem, quantitative protease sensitivity assays have been used to assess the ability of analogs to bind VDR (Gardezi et al., 2001; Kittaka et al., 2003). The inability of a ligand to stabilize a receptor conformation does not necessarily reflect a lack of affinity for VDR, but the ability to stabilize a conformation does reflect effective binding. In the present study, protease sensitivity assays have been used to evaluate the ability of 1α,25(OH)$_2$D$_3$ to induce conformational changes in wild-type and variant VDRs upon binding. Partial trypsin digestion of VDR, in the presence or absence of saturating concentrations of 1α,25(OH)$_2$D$_3$, has revealed that LBD is much more resistant to proteolysis in the presence of hormone than in the absence. In the absence of the ligand, the receptor is rapidly degraded. However, following the addition of hormone, digestion with trypsin resulted in distinct proteolytic fragments corresponding to molecular masses of approximately 30 kDa and 20.1 kDa. The 30 kDa ligand-protected fragment has been shown to originate from the steroid binding region of the VDR (Väisänen et al., 1997). These results indicate that the trypsin cleavage sites are

more accessible in the apoprotein conformation than the holoprotein. Hormone binding changes the conformation of VDR leading to increased stability towards limited proteolytic digestion. Ligand-binding induced conformational changes of the receptor, detected by limited proteolytic analysis, have been previously reported by other research groups (Peleg et al., 1995; Väisänen et al.,1998) with similar results. However, the principle trypsin-resistant fragment reported by other groups is 34 kDa in size, and, in the present study, it is 30 kDa.

Previously reported results of trypsin sensitivity assays for the mutant VDRs, S237A and R274A, have shown complete digestion of the 34 kDa fragment in the presence of 1μM concentration of $1\alpha,25(OH)_2D_3$ (Väisänen et al., 1998; Väisänen et al., 1999). However, in the present study, the 30 kDa fragment obtained following digestion with trypsin was visible for all three mutant VDRs, irrespective of the presence or absence of the hormone. The residue at the 274 position in VDR is an arginine, a trypsin cutting site, which is replaced with an alanine in the R274A VDR mutant. However, this residue is located within the hydrophobic pocket of the LBD and, therefore, may not be accessible for trypsin digestion. There are three possible explanations for the similarity in the pattern of digestion fragments for the results from the present study in the presence and absence of the ligand: 1. the decreased ability, or inability, of mutant VDRs to bind $1\alpha,25(OH)_2D_3$ at the concentrations used, 2. ligand-induced conformational changes of the mutant VDRs differ from wild-type VDR, and 3. unliganded mutant VDRs have different conformations than wild-type VDR.

In conclusion, the results from ligand saturation binding assays, taken together

with the results from protease sensitivity assays, are important for a better understanding of the interaction effects between 1α,25$(OH)_2D_3$ and the 1α-hydroxy group contact residues, S237 and R274, of the VDR. The results from the present study further enhance and reinforce the findings of other research groups (Kristjansson et al., 1993; Yamamoto et al., 2000; Choi et al., 2003; Gardezi et al., 2001) that confirm the importance of the 1α-hydroxyl group of 1α,25$(OH)_2D_3$, in addition to the importance of residues, S237 and R274, for proper functioning of the VDR. Future experiments include transcription activation analysis of the mutant receptors to study the effect of the mutations on regulation of vitamin D-sensitive genes. Also, studies of additional mutants, such as substitution of R274 with another residue of similar size that can abolish hydrogen bonding with the ligand, can contribute to a better understanding of the role of spatial requirements for ligand binding. Knowledge gained from the results of such studies may be important towards the development of vitamin D analogs with therapeutic potential in the management or prevention of rickets, osteoporosis, psoriasis, cancer, and immune-mediated diseases.

SUMMARY

The primary focus of this research was to investigate the structure-function relationship of the VDR, which is important for a better understanding of the mechanism of action of $1\alpha,25(OH)_2D_3$ mediated by the VDR. The importance of amino acid residues S237 and R274 that form hydrogen bonding interactions with the biologically important 1α-OH group of $1\alpha,25(OH)_2D_3$, were analyzed. Site-directed mutagenesis was used to construct the three mutant VDRs: S237A, R274A, and S237A/R274A. The cDNA of wild-type or mutant VDRs was transfected into COS-7 cells or FreeStyle™ 293-F cells to evaluate the relative binding affinities of the wild-type and variant VDRs. The binding data obtained with FreeStyle™ 293-F cells were found to have much better signal-to-noise ratio in comparison with that obtained from COS-7 cells. Data from the ligand saturation binding assays indicate S237 to be significant but not essential for binding, unlike R274, which is absolutely essential. This explains the undetectable binding with the double mutant, S237A/R274A. Results from protease sensitivity assays show that treatment with $1\alpha,25(OH)_2D_3$ protected the receptor against partial proteolysis, indicating holo- and apo- receptors have different conformations. The three possible explanations for the similarity in the pattern of digestion fragments in the presence and absence of the ligand for the mutant VDRs include: the decreased ability, or inability, of mutant VDRs to bind $1\alpha,25(OH)_2D_3$ at the concentrations used, ligand-induced conformational changes of the mutant VDRs differ from wild-type VDR, and unliganded mutant VDRs have different conformations than wild-type VDR.

REFERENCES

Abe, E., Ishimi, Y., Takahashi, N., Akatsu, T., Ozawa, H., Yamana, H., Yoshiki, S., Suda, T. (1988) A differentiation-inducing factor produced by the osteoblastic cell line MC3T3-E1 stimulates bone resorption by promoting osteoclast formation. *J. Bone Miner. Res. 3*, 635-645.

Abe, E., Miyaura, H., Sakagami, M., Takeda, K., Konno, T., Yamazaki, S., Yoshiki, S., Suda, T. (1981) Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. USA 78*, 4990-4994.

Acevedo, A., Stoynova, L., Davis, K., Solórzano, Collins, E.D. (2004) Role of residues 143 and 278 of the human nuclear vitamin D receptor in the full-length and Δ165-215 deletion mutant. *J. Steroid Biochem. Mol. Biol. 89-90*, 83-87.

Allan, G.F., Leng, X., Tsai, S.Y., Weigel, N.L., Edwards, D.P., Tsai, M.J., O'Malley, B.W. (1992) Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. *J. Biol. Chem. 267*, 19513-19520.

Aranda, A., and Pascual A. (2001) Nuclear hormone receptors and gene expression. *Phys. Rev. 81*, 1269-1304.

Ausbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Eds. (1997) Detection of nucleic acids using absorption spectroscopy. In: *Short protocols in Molecular Biology* (3rd edition), Wiley & Sons, Inc.: New York, pp A3/11-A3/12.

Axén, E., Bergman, T., Wikvall, K. (1994) Microsomal 25-hydroxylation of vitamin D_2 and vitamin D_3 in pig liver. *J. Steroid Biochem. Mol. Biol. 51*, 97-106.

Baker, A.R., McDonnell, D.P., Hughes, M., Crisp, T.M., Mangelsdorf, D.J., Haussler, M.R., Pike, J.W., Shine, J., O'Malley, B.W. (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA 85*, 3294-3298.

Baran, D.T., Sorensen, A.M., Honeyman, T.W., Ray, R., Holick, M.F. (1989) Rapid actions of 1 alpha,25-dihydroxyvitamin D_3 on Ca^{2+} and phospholipids in isolated rat liver nuclei. *FEBS Lett. 259*, 205-208.

Baran, D.T., Sorenson, A.M., Honeyman, R.W., Ray, R., Holick, M.F. (1990) 1 alpha,25-dihydroxyvitamin D_3-induced increments in hepatocyte cytosolic calcium and

lysophosphatidylinositol: inhibition by pertussis toxin and 1 beta,25-dihydroxyvitamin D_3. *J. Bone Miner. Res. 5*, 517-524.

Bhalla, A.K., Amento, E.P., Krane, S.M. (1986) Differential effects of 1,25-dihydroxyvitamin D_3 on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. *Cell. Immunol. 98*, 311-322.

Bhalla, A.K., Amento, E.P., Serog, B., Glimcher, L.H. (1984) 1,25-Dihydroxyvitamin D_3 inhibits antigen-induced T cell activation. *J. Immunol. 133*, 1748-1754.

Bissonnette, M., Tien, X.-Y., Niedziela, S. M., Hartmann, S.C., Frawley, B.P., Jr., Roy, H.K., Sitrin, M.D., Perlman, R.L., Brasitus, T.A. (1994) $1,25(OH)_2$ vitamin D_3 activates PKC-alpha in Caco-2 cells: a mechanism to limit secosteroid-induced rise in $[Ca^{+2}]_i$. *Am. J. Physiol. 267*, G465-G475.

Blackmore, P.F., Beebe, S.J., Danforth, D.R., Alexander, N. (1990) Progesterone and 17 alpha-progesterone. Novel stimulators of calcium influx in human sperm. *J. Biol. Chem. 265*, 1376-1380.

Blutt, S.E., McDonnell, T.J., Polek, T.C., Weigel, N.L. (2000) Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. *Endocrinology 141*, 10-17.

Bouillon, R., Okamura, W.H., Norman, A.W. (1995) Structure-function relationships in the vitamin D endocrine system. *Endocr Rev. 16*, 200-257.

Bronner, F., Pansu, D., Stein, W.D. (1986) An analysis of intestinal calcium transport across the rat intestine. *Am. J. Physiol. 250*, G561-G569.

Cade, C., and Norman, A.W. (1986) Vitamin D_3 improves impaired glucose tolerance and insulin secretion in the vitamin D-deficient rat *in vivo*. *Endocrinology 119*, 84-90.

Caffery, J.M., and Farach-Carson, M.C. (1989) Vitamin D3 metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. *J. Biol. Chem. 264*, 20265-20274.

Canalejo, A., Almaden, Y., Torregrosa, V., Gomez-Villamandos, J.C., Ramos, B., Campistol, J.M., Felsenfeld, A.J., Rodriguez, M. (2000) The in vitro effect of calcitriol on parathyroid cell proliferation and apoptosis. *J. Am. Soc. Nephrol. 11*, 1865-1872.

Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L.J., Grippo, J.F., Hunziker, W. (1993) Two nuclear signalling pathways for vitamin D. *Nature, 361*, 657-660.

Chiu, K.C., Chu, A., Go, V.L., Saad, M.F. (2004) Hypovitaminosis D is associated with

insulin resistance and beta cell dysfunction. *Am. J. Clin. Nutr. 79*, 820-825.

Choi, M., Yamamoto, K., Itoh, T., Makishima, M., Mangelsdorf, D.J., Moras, D., DeLuca, H.F., Yamada, S. (2003) Interaction between vitamin D receptor and vitamin D ligands: two-dimensional alanine scanning mutational analysis. *Chem. Biol. 10*, 261-270.

Choi, M., Yamamoto, K., Masuno, H., Nakashima, K., Taga, T., Yamada, S. (2001) Ligand recognition by the vitamin D receptor. *Bioorg. Med. Chem. 9*, 1721-1730.

Collins, E.D. (2000) Molecular biology of the vitamin D endocrine system. *Recent Res. Devel. Steroid Biochem. Mol. Biol. 1*, 81-106.

Collins, E.D. and Norman, A.W. (2001) Vitamin D. In: *Handbook of Vitamins* (3rd edition), Rucker, R., Suttie, J., McCormick, D., Machlin, L. Eds., Marcel Dekker, Inc.: New York, pp 51-113.

Crowfoot, D. and Dunitz, J.D. (1948) Structure of calciferol. *Nature 162*, 608.

Daiger, S.P., Schanfield, M.S., Cavalli-Sforza, L.L. (1975) Group-specific component (Gc) proteins bind vitamin D and 25-hydroxyvitamin D. *Proc. Natl. Acad. Sci. USA 72*, 2076-2080.

Darwish, H.M., Burmester, J.K., Moss, V.E., DeLuca, H.F. (1993) Phosphorylation is involved in transcriptional activation by the 1,25-dihydroxyvitamin D_3 receptor. *Biochem. Biophys. Acta. 1167*, 29-36.

DeBoland, A.R., and Norman, A.W. (1990) Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D_3-dependent transcaltachia (the rapid stimulation of duodenal Ca^{2+} transport). *Endocrinology 127*, 2475-2480.

DeBoland, A.R., Nemere, I., Norman, A.W. (1990) Ca^{2+}-channel agonist Bay K8644 mimics 1α,25$(OH)_2$-vitamin D_3 rapid enhancement of Ca^{2+} transport in chick perfused duodenum. *Biochem. Biophys. Res. Commun. 166*, 217-222.

Demay, M.B., Gerardi, J.M., DeLuca, H.F., Kronenberg, H.M. (1990) DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. USA 87*, 369-373.

Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science 240*, 889-895.

Feldman, D., and Malloy, P.J. (1990) Hereditary 1,25-dihydroxyvitamin D resistant rickets: molecular basis and implications for the role of 1,25$(OH)_2D_3$ in normal physiology. *Mol. Cell. Endocrinol. 72*, C57-C62.

Fleet, J.C., and Wood, R.J. (1999) Specific $1,25(OH)_2D_3$-mediated regulation of transcellular calcium transport in Caco-2 cells. *Am. J. Physiol. 276*, G958-G964.

Forman, B.M, and Samuels, H.H. ((1990) Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol. 4*, 1293-1301.

Fraser, D.R. and Kodicek, E. (1970) Unique biosynthesis by kidney of a biological active vitamin D metabolite. *Nature 228*, 764-766.

Gametchu, B., Watson, C.S., Wu, S. (1993) Use of receptor antibodies to demonstrate membrane glucocorticoid receptor in cells from human leukemic patients. *FASEB J. 7*, 1283-1292.

Gardezi, S. A., Nguyen, C., Malloy, P.J., Posner, G.H., Feldman, D., Peleg, S. (2001) A rationale for treatment of hereditary vitamin D-resistant rickets with analogs of 1 alpha,25-dihydroxyvitamin D(3). *J. Biol. Chem. 276*, 29148-29156.

Gedik, O., and Akalin, S. (1986) Effects of vitamin D deficiency and repletion on insulin and glucagon secretion in man. *Diabetologia 29*, 142-145.

Ghazarian, J.G., Jefcoate, C.R., Knutson, J.C., Orme-Johnson, W.H., DeLuca, H.F. (1974) Mitochondrial cytpchrome P_{450}. A component of chick kidney 25-hydroxycholecalciferol-1 alpha-hydroxylase. *J. Biol. Chem. 249*, 3026-3033.

Golovko, O, Nazarova, N., Tuohimaa, P. (2005) Vitamin D-induced up-regulation of tumor necrosis factor alpha (TNF-alpha) in prostate cancer cells. *Life Sci. 77*, 562-577.

Green, S., and Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet. 4*, 309-314.

Gregori, S., Casorati, M., Amuchastegui, S., Smiroldo, S., Davalli, A.M., Adorini, L. (2001) Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D_3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J. Immunol. 167*, 1945-1953.

Griffin, M.D., Xing, N., Kumar, R. (2003) Vitamin D and its analogs as regulators of immune activation and antigen presentation. *Annu. Rev. Nutr. 23*, 117-145.

Haddad, J.G., Harper, K.D., Gouth, M., Pietra, G.G., Sanger, J.W. (1990) Angiopathic consequences of saturating the plasma scavenger system for actin. *Proc. Natl. Acad. Sci. USA 87*, 1381-1385.

Ham, J., and Parker, M.G. (1989) Regulation of gene expression by nuclear hormone receptors. *Curr. Opin. Cell Biol. 1*, 503-511.

Hannah, S.S., and Norman, A.W. (1994) 1 alpha,25-dihydroxyvitamin D_3-regulated expression of the eukaryotic genome. *Nutr. Rev. 52*, 376-382.

Hansen, C.M, Binderup, L., Hamberg, K.J., Carlberg, C. (2001) Vitamin D and cancer: Effects of $1,25(OH)_2D_3$ and its analgs on growth control and tumorigenesis. *Front. In Biosci. 6*, D820-848.

Harrison, J.R., Peterson, D.N., Lichtler, A.C., Mador, A.T., Rowe, D.W., Kream, B.E. (1989) 1,25-Dihydroxyvitamin D3 inhibits transcription of type I collagen genes in the rat osteosarcoma cell line ROS 17/2.8. *Endocrinology 125*, 327-333.

Haussler, M.R., Haussler, C.A., Jurutka, P.W., Thompson, P.D., Hsieh, J.C., Remus, L.S., Selznick, S.H., Whitfield, G.K. (1997) The vitamin D hormone and its nuclear receptor: molecular actions and disease states. *J. Endocrinol.154*, 557-573.

Haussler, M.R., Mangelsdorf, D.J., Komm, B.S., Terpening, C.M., Yamaoka, K., Allegreto, E.A., Baker, A.R., Shine, J., McDonnell, D.P., Hughes, M., Weigel, N.L., O'Malley, B.W., Pike, J.W. (1988) Molecular biology of the vitamin D hormone. *Recent Prog. Horm. Res. 44*, 263-305.

Heberden, C., Denis, I., Pointillart, A., Mercier, T. (1998) TGF-beta and calcitriol. *Gen. Pharmacol. 30*, 145-151.

Henry, H.L. (1992) Vitamin D hydroxylases. *J. Cell. Biochem. 49*, 4-9.

Henry, H.L. and Norman, A.W. (1974) Studies on calciferol metabolism. IX. Renal 25-hydroxy-vitamin D_3-1 hydroxylase. Involvement of cytochrome P-450 and other properties. *J. Biol. Chem. 249*, 7529-7535.

Henry, H.L. and Norman, A.W. (1978) Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. *Science 201*, 835-837.

Henry, H.L., Midgett, R.J., Norman, A.W. (1974) Regulation of 25-hydroxyvitamin D_3-1-hydroxylase *in vivo*. *J. Biol. Chem. 249*, 7584-7592.

Hodgkin, D.C., Rimmer, B.M., Dunitz, J.D., Trueblood, K.N. (1963) The crystal structure of a calciferol derivative. *J Chem Soc 947*, 4945.

Holick, M.F. (1981) The cutaneous photosynthesis of previtamin D_3: a unique photoendocrine system. *J. Invest. Dermatol. 77*, 51-58.

Holick, M.F., Schnoes, H.K., DeLuca, H.F., Suda, T., Cousins, R.J. (1971) Isolation and identification of 1,25-dihydroxycholecalciferol. A metabolite of vitamin D active in intestine. *Biochemistry 10*, 2799-2804.

Ishizuka, S., Ohba, T., Norman, A.W. (1988) 1α,25$(OH)_2D_3$-26,23-Lactone is a major metabolite of 1α,25$(OH)_2D_3$ under physiological conditions, in *Vitamin D: Molecular Cellular and Clinical Endocrinology* (Norman, A.W., Schaefer, K., Grigoleit, H.G., Herrath, D.V., eds.), Walter de Gruyter, Berlin, pp. 143-144.

Issa, L.L., Leong, G.M., Eisman, J.A. (1998) Molecular mechanism of vitamin D receptor action. *Inflamm. Res. 47*, 451-475.

Jones, G., Strugnell, S.A., DeLuca, H.F. (1998) Current understanding of the molecular actions of vitamin D. *Physiol Rev. 78*, 1193-1231.

Jordan, S.C., Toyada, M., Prehn, J, Lemire, J.M., Sakai, R., Adams, J.S. (1989) 1,25-Dihydroxyvitamin-D_3 regulation of interleukin-2 and interleukin-2 receptor levels and gene expression in human T cells. *Mol. Immunol. 10,* 979-984.

Jurutka, P.W., Hsieh, J.C., Haussler, M.R. (1993) Phosphorylation of the human 1,25-dihydroxyvitamin D_3 receptor by cAMP-dependent protein kinase, *in vitro*, and in transfected COS-7 cells. *Biochem. Biophys. Res. Commun. 191*, 1089-1096.

Karbach, U. (1992) Paracellular calcium transport across the small intestine. *J. Nutr. 122*, 672-677.

Kerner, S.A., Scott, R.A., Pike, J.W. (1989) Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3. *Proc. Natl. Acad. Sci. USA 86*, 4455-4459.

Khare, S., Tien, X,-Y, Wilson, D., Wali, R.K., Bissonnette, B.M., Scaglione-Sewell, B., Sitrin, M.D., Brasitus, T.A. (1994) The role of protein kinase C-alpha in the activation of particulate guanylate cyclase by 1 alpha,25-dihydroxyvitamin D_3 in CaCo-2 cells. *Endocrinology 135*, 277-283.

Kittaka, A., Kurihara, M., Peleg, S., Suhara, Y., Takayama, H. (2003) 2 alpha-(3-hydroxypropyl)- and 2 alpha-(3-hydroxypropoxy)-1 alpha,25-Dihydroxyvitamin D_3 accessible to vitamin D receptor mutant related to hereditary vitamin D-resistant rickets. *Chem. Pharm. Bull. 51*, 357-358.

Koeffler, H.P., Amatruda, T., Ikekawa, N., Kobayashi, Y., DeLuca, H.F. (1984) Induction of macrophage differentiation of human normal and leukemic myeloid stem cells by 1,25-dihydroxyvitamin D_3 and its fluorinated analogs. *Cancer Res. 44*, 5624-5628.

Koenig, H., Fan, C.C, Goldstone, A.D., Lu, C.Y., Trout, J.J. (1989) Polyamines mediate androgenic stimulation of calcium fluxes and membrane transport in rat heart myocytes. *Circ. Res. 64*, 415-426.

Kragballe, K., Beck, H.I., Sogaard, H. (1988) Improvement of psoriasis by a topical vitamin D_3 analogue (MC 903) in a double-blind study. *Br. J. Dermatol. 119*, 223-230.

Kristjansson, K., Rut, A.R., Hewison, M., O'Riordan, J.L., Hughes, M.R. (1993) Two mutations in the hormone binding domain of the vitamin D receptor cause tissue resistance to 1,25 dihydroxyvitamin D_3. *J. Clin. Invest. 92*, 12-16.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.M., Chambon, P. (1986) The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *Embo. J. 5*, 891-897.

Kutuzova, G.D., and DeLuca, H.F. (2004) Gene expression profiles in rat intestine identify pathways for 1,25-dihydroxyvitamin D(3) stimulated calcium absorption and clarify its immunomodulatory properties. *Arch. Biochem. Biophys. 432*, 152-166.

Lawson, D.E., Fraser, D.R., Kodicek, E., Morris, H.R., Williams, D.H. (1971) Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. *Nature 230*, 228-230.

Lee, E., Jeon, S.H., Yi, J.Y., Jin, Y.J., Son, Y.S. (2001) Calcipotriol inhibits autocrine phosphorylation of EGF receptor in a calcium-dependent manner, a possible mechanism for its inhibition of cell proliferation and stimulation of cell differentiation. *Biochem. Biophys. Res. Commun. 284*, 419-425.

Lee, W.M. and Galbraith, R. M. (1992) The extracellular actin-scavenger system and actin toxicity. *N. Engl. J. Med. 326*, 1335-1341.

Li, Y.C., Kong, J., Wei, M., Chen, Z.F., Liu, S.Q., Cao, L.P. (2002) 1,25-Dihydroxyvitamin D_3 is a negative endocrine regulator of the renin-angiotensin system. *J. Clin. Invest. 110*, 229-238.

Lieberherr, M. (1987) Effects of vitamin D3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. *J. Biol. Chem. 262*, 13168-13173.

Liu, M., Lee, M.H., Cohen, M., Bommakanti, M., Freedman, L.P. (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev. 10, 142*-153.

Lowe, K.E., Maiyar, A.C., Norman A.W. (1992) Vitamin D-mediated gene expression. *Crit Rev Eukaryot Gene Expr. 2,* 65-109.

Lyakhovich, A., Aksenov, N., Miettinen, S., Ahonen, M.H., Syvälä, H., Ylikomi, T., Tuohimaa, P. (2000) Vitamin D induced up-regulation of keratinocyte growth factor (FGF-7/KGF) in MCF-7 human breast cancer cells. *Biochem. Biophys. Res. Commun.*

273, 675-680.

Majewska, M.D. and Vaupel, D.B. (1991) Steroid control of uterine motility via gamma-aminobutyric acidA receptors in the rabbit: a novel mechanism? *J. Endocrinol. 131*, 427-434.

Mangelsdorf, D.J., and Evans, R.M. (1995) The RXR heterodimers and orphan receptors. *Cell 83*, 841-850.

Mantell, D.J., Owens, P.E., Bundred, N.J., Mawer, E. B., Canfield, A.E. (2000) 1 alpha,25-dihydroxyvitamin D(3) inhibits angiogenesis in vitro and in vivo. *Circ. Res. 87*, 214-220.

Mathieu, C., van Etten, E., Decallonne, B., Gysemans, C., Bouillon, r., Overbergh, L. (2004) Vitamin D and 1,25-dihydroxyvitamin D_3 as modulators in the immune system. *J. Steroid Biochem. Mol. Biol. 89-90*, 449-452.

Mawer, E.B. (1980) Clinical implications of measurements of circulating vitamin D metabolites. *Clin. Endocrinol. Metab. 9*, 63-79.

McCarthy, D.M., San Miguel, J.F., Freake, H.C., Green, P.M., Zola, H., Catovsky, D., Goldman, J.M. (1983) 1,25-dihydroxy vitamin D_3 inhibits proliferation of human promyelocytic (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. *Leuk. Res. 7*, 51-55.

McCutchan, J.H., and Pagano, J.S. (1968) Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer Inst. 41*, 351-357.

McDonnell, D.P., Mangelsdorf, D.J., Pike, J.W., Haussler, M.R., O'Malley, B.W. (1987) Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science 235*, 1214-1217.

Meikle, M.C. (1988) Bone Physiology. Hypercalcaemia of malignancy. *Nature 336*, 311.

Mendoza, C. and Tesarik, J. (1993) A plasma-membrane progesterone receptor in human sperm is switched on by increasing intracellular free calcium. *FEBS Lett. 330*, 57-60.

Miller, W.L., and Portale, A.A. (2003) Vitamin D biosynthesis and vitamin D 1 alpha-hydroxylase deficiency. *Endocr. Dev. 6*, 156-174.

Morley, P., Whitfield, J.F., Vanderhyden, B.C., Tsang, B.K., Schwartz, J.L. (1992) A

new, nongenomic estrogen action: the rapid release of intracellular calcium. *Endocrinology 131*, 1305-1312.

Murao, S., Gemmell, M.A., Callaham, M.F., Anderson, N.L., Huberman, E. (1983) Control of macrophage cell differentiation in human promyelocytic HL-60 leukemia cells by 1,25-dihydroxyvitamin D_3 and phorbol-12-myristate-13-acetate. *Cancer Res. 43*, 4989-4996.

Naveh-Many, T., Marx, R., Keshet, E., Pike, J.W., Silver, J. (1990) Regulation of 1,25-dihydroxyvitamin D_3 receptor gene expression by 1,25-dihydroxyvitamin D_3 in the parathyroid in vivo. *J. Clin. Invest. 86*, 1968-1975.

Nayeri, S., and Carlberg, C. (1997) Functional conformations of the nuclear 1 alpha,25-dihydroxyvitamin D_3 receptor. *Biochem. J. 327*, 561-568.

Nemere, I. (1992) Vesicular calcium transort in chick intestine. *J. Nutr. 122*, 657-661.

Nemere, I., And Norman, A.W. (1987) Rapid action of 1,25-dihydroxyvitamin D3 on calcium transport in perfused chick duodenum: effect of inhibitors. *J. Bone. Miner. Res. 2*, 99-107.

Nemere, I., Dormanen, M.C., Hammond, M.W., Okamura, W. H., Norman, A.W. (1994) Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *J. Biol. Chem. 269*, 23750-23756.

Nemere, I., Farach-Carson, M.C., Rohe, B., Sterling, T.M., Norman, A.W., Boyan, B.D., Safford, S.E. (2004) Ribozyme knockdown functionally links a $1,25(OH)_2D_3$ membrane binding protein ($1,25D_3$-MARRS) and phosphate uptake in intestinal cells. *Proc. Natl. Acad. Sci. USA 101*, 7392-7397.

Nemere, I., Leathers, V., Norman, A.W. (1986) 1,25-dihydroxyvitamin D_3- mediated intestinal calcium transport. Biochemical indentification of lysosomes containing calcium and calcium-binding protein (calbindin-D28K). *J. Biol. Chem. 261*, 16106-16114.

Nemere, I., Yoshimoto, Y., Norman, A.W. (1984) Calcium transport in perfused duodena from normal chicks: enhancement within fourteen minutes of exposure to 1,25-dihydroxyvitamin D_3. *Endocrinology 115*, 1476-1483.

Nguyen, T.M., Lieberherr, M., Fritsch, J., Guillozo, H., Alvarez, M.L., Fitouri, Z., Jehan, F., le Garabedian, M. (2003) The rapid effects of 1,25-dihydroxyvitamin D_3 require the vitamin D receptor and influence 24-hydroxylase activity: studies in human skin fibroblasts bearing vitamin D receptor mutations. *J. Biol. Chem. 279*, 7591-7597.

Nickerson, T., and Huynh, H. (1999) Vitamin D analogue EB 1089-induced prostate regression is associated with increased gene expression of insulin-like growth factor binding proteins. *J. Endocrinol. 160*, 223-229.

Norman, A.W., Frankel, J.B., Heldt, A.M., Grodsky, G.M. (1980) Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science 209*, 823-825.

Norman, A.W. (1979) *Vitamin D: The Calcium Homeostatic Steroid Hormone*, Academic Press, New York, pp 1-490.

Norman, A.W., Adams, D., Collins, E.D., Okamura, W.H., Fletterick, R.J. (1999) Three-dimensional model of the ligand binding domain of the nuclear receptor for 1alpha,25-dihydroxy-vitamin D(3). *J. Cell. Biochem. 74*, 323-333.

Norman, A.W., Bishop, J.E., Collins, E.D., Seo, E.G., Satchell, D.P., Dormanen, M.C., Zanello, S.B., Farach-Carson, M.C., Bouillon, R., Okamura, W.H. (1996) Differing shapes of 1 alpha,25-dihydroxyvitamin D3 function as ligands for the D-binding protein, nuclear receptor and membrane receptor: a status report. *J. Steroid Biochem. Molec. Biol. 56*, 13-22.

Norman, A.W., Leathers V., Bishop, J.E. (1983) Normal egg hatchability requires the simultaneous administration to the hen of 1 alpha,25-dihydroxycholecalciferol and 24R,25-dihydroxycholecalciferol. *J. Nutr. 113*, 2505-2515.

Norman, A.W., Myrtle, J.F., Midgett, R.J., Nowicki, H.G., Williams, V., Popjak, G. (1971) 1,25-dihydroxycholecalciferol: identification of the proposed active form of vitamin D3 in the intestine. *Science 173*, 51-54.

Norman, A.W., Nemere, I., Williams, G., King, M. (1984) 1α,25-dihydroxyvitamin D_3 mediates biological responses both as a steroid hormone and as a membrane-active agent. In: Endocrine Control of Bone and Calcium Metabolism (Cohn, D.V., Potts, J.T., Jr., Fujita, T., eds) Ed. 1, pp. 316-319. Elsevier Science, The Netherlands.

Okamura, W.H., Do, S., Kim, H., Tan, E., Tham, F., Jeganathan, S., Ferrero, M., Norman, A.W. (2000) Constitutional and conformational isomers of 1 alpha,25-dihydroxyvitamin D_3 and its metabolites. In: Norman, A.W., Bouillon, R., Thomasset, M., editors. Vitamin D endocrine system: Structural, biological, genetic, and clinical aspects. Riverside: Univ. California-Riverside Printing and Reprographics. pp 11-22.

Okamura, W.H., Norman, A.W., Wing, R.M. (1974) Vitamin D: concerning the relationship between molecular topology and biological function. *Proc. Natl. Acad. Sci. USA 71*, 4194-4197.

Okamura, W.H., Palenzuela J.A., Plumet, J., Midland, M.M. (1992) Vitamin D:

structure-function analyses and the design of analogs. *J. Cell. Biochem 49*, 10-18.

Orchinik, M., Murray, T.F., Moore, F.L. (1991) A corticosteroid receptor in neuronal membranes. *Science 252*, 1848-1851.

Otterbien, L.R., Cosio, C., Graceffa, P., Dominguez, R. (2002) Crystal structures of the vitamin D-binding protein and its complex with actin: structural basis of the actin-scavenger system. *Proc. Natl. Acad. Sci. USA 99,* 8003-8008.

Palmer, H.G., Larriba, M.J., Garcia, J.M., Ordonez-Moran, P., Pena, C., Peiro, S., Puig, I., Rodriguez, R., de la Fuente, R., Bernad, A., Pollan, M., Bonilla, F., Gamallo, C., de Herreros, A.G., Munoz, A. (2004) The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. *Nat. Med. 10*, 917-919.

Pan, L.C., and Price, P.A. (1984) The effect of transcriptional inhibitors on the bone gamma- carboxyglutamic acid protein response to 1,25-dihydroxyvitamin D_3 in osteosarcoma cells. *J. Biol. Chem. 259*, 5844-5847.

Park, W.H., Seol, J.G., Kim, E.S., Hyun, J.M., Jung, C.W., Lee, C.C., Binderup, L., Koeffler, H.P., Kim, B.K., Lee, Y.Y. (2000). Induction of apoptosis by vitamin D3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase. *Br. J. Haematol. 109*, 576-583.

Peleg, S., Shastry, M., Collins, E.D., Bishop, J.E., Norman, A.W. (1995) Distinct conformational changes induced by 20-epi analogues of 1 alpha,25-dihydroxyvitamin D3 are associated with enhanced activation of the vitamin D receptor. *J. Biol. Chem. 270*, 10551-10558.

Pettifor, J.M., Ross, F.P., Wang, J. (1977) Serum levels of 25-hydroxycholecalciferol as a diagnostic aid in vitamin D deficiency states. *S. Afr. Med. J. 51*, 580-583.

Rastinejad, F., Perlmann, T., Evans, R.M., Sigler, P.B. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature, 375*, 203-211.

Redaelli, C.A., Wagner, M., Gunter-Duwe, D., Tian, Y.H., Stahel., P.F., Mazzucchelli, L., Schmid, R.A, Schilling, M.K. (2002) 1alpha,25-dihydroxyvitamin D_3 shows strong and additive immunomodulatory effects with cyclosporine A in rat renal allotransplants. *Kidney Int. 61*, 288-296.

Rehberger, P., Rexin, M., Gehring, U. (1992) Heterotetrameric structure of the human progesterone receptor. *Proc. Natl. Acad. Sci. USA. 89*, 8001-8005.

Rigby, W.F., Noelle, R.J., Krause, K, Fanger, M.W. (1985) The effects of 1,25-dihydroxyvitamin D_3 on human T lymphocyte activation and proliferation: a cell cycle

analysis. *J. Immunol. 135*, 2279-2286.

Rigby, W.F., Stacy, T., Fanger, M.W. (1984) Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D_3 (calcitriol). *J. Clin. Invest. 74*, 1451-1455.

Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B., Moras, D. (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol. Cell. 5*, 173-179.

Roodman, G.D., Ibbotson, K.J., MacDonald, B.R., Kuehl, T.J., Mundy, G.R. (1985) 1,25-dihydroxyvitamin D3 causes formation of multinucleated cells with several osteoclast characteristics in cultures of primate marrow. *Proc. Natl. Acad. Sci. USA 82*, 8213-8217.

Rowe, D.W., and Kream, B.E. (1982) Regulation of collagen synthesis in fetal rat calvaria by 1,25-dihydroxyvitamin D_3. *J. Biol. Chem. 257*, 8009-8015.

Rozen, F., and Pollak, M. (1999) Inhibition of insulin-like growth factor I receptor signaling by the vitamin D analog, KB1089 in MCF-7 breast cancer cells: A role for insulin-like growth factor binding proteins. *Int. J. Oncol. 15*,589-594.

Russell, J., Lettieri, D., Sherwood, L.M. (1986) Suppression by $1,25(OH)_2D_3$ of transcription of the pre-proparathyroid hormone gene. *Endocrinology 119*, 2864-2866.

Saarem, K., Bergseth, S., Oftebro, H., Pedersen, J.I. (1984) Subcellular localization of vitamin D_3 25-hydroxylase in human liver. *J. Biol. Chem. 259*, 10936-10940.

Schachter, D., and Kowarski, S. (1982) Isolation of the protein IMCal, a vitamin D-dependent membrane component of the intestinal transport mechanism for calcium. *Fed. Proc. 41*, 84-87.

Schule R., Umesono, K., Mangelsdorf, D.J., Bolado, J., Pike, J.W., Evans, R.M. (1990) Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. *Cell 61*, 497-504.

Segal, J. (1990) Thyroid hormone action at the level of plasma membrane. *Thyroid 1*, 83-87.

Shaffer, P.L., and Gewirth, D.T. (2002) Structural basis of VDR-DNA interactions on direct repeat response elements. *The Embo. J. 21*, 2242-2252.

Shaffer, P.L., McDonnell, D.P., Gewirth, D.T. (2005) Characterization of transcriptional activation and DNA-binding functions in the hinge region of the vitamin D receptor. *Biochemistry 44*, 2678-2685.

Shinki, T., Jin, C.H., Nishimura, A., Nagai, Y., Ohyama Y., Noshiro, M., Okuda, K., Suda, T. (1992) Parathyroid hormone inhibits 25-hydroxyvitamin D3-24-hydroxylase mRNA expression stimulated by 1 alpha,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. *J. Biol. Chem. 267*, 13757-13762.

Silver, J. and Kronenberg, H.M. (1996) Parathyroid hormone – molecular biology and regulation. In: Principles of Bone Biology, Academic Press, Inc. Eds., Bilezikian, J.P., Raisz, L.G., Rodan, G.A., pp 325-335.

Silver, J., Naveh-Many, T., Mayer, H., Schmelzer, H.J., Popovtzer, M.M. (1986) Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat. *J. Clin. Invest. 78*, 1296-1301.

Slater, S.J., Kelly, M.B., Taddeo, F.J., Larkin, J.D., Yeager, M.D., McLane, J.A., Ho, C., Stubbs, C.D. (1995) Direct activation of protein kinase C by 1 alpha,25-dihydroxyvitamin D_3. *J. Biol. Chem. 270*, 6639-6643.

Smith, T.J., Davis, F.B., Davis, P.J. (1992) Stereochemical requirements for the modulation by retinoic acid of thyroid hormone activation of Ca(2+)-ATPase and binding at the human erythrocyte membrane. *Biochem. J. 284*, 583-587.

Stene, L.C., Joner, G; Norwegian Childhood Diabetes Study Group. (2003) Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type I diabetes: a large population-based, case-control study. *Am. J. Clin. Nutr. 78*, 1128-1134.

Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J.M., Martin, T.J., Suda, T. (1988) Osteoblastic cells are involved in osteoclast formation. *Endocrinology 123*, 2600-2602.

Tucker, G 3rd., Gagnon, R.E., Haussler, M.R. (1973) Vitamin D 3 -25-hydroxylase: tissue occurrence and apparent lack of regulation. *Arch. Biochem. Biophys. 155*, 47-57.

Umesono, K., Murakami, K.K., Thompson, C.C., Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell 65*, 1255-1266.

Väisänen, S., Douchier, C., Rouvinen, J., Mäenpää, P.H. (1999) The importance of the putative helices 4 and 5 of human vitamin D(3) receptor for conformation and ligand binding. *Biochem. Biophys. Res. Commun. 264*, 478-482.

Väisänen, S., Juntunen, K., Itkonen, A., Vihko, P., Mäenpää, P.H. (1997) Conformational studies of human vitamin-D receptor by antipeptide antibodies, partial proteolytic digestion and ligand binding. *Eur. J. Biochem. 248*, 156-162.

Väisänen, S., Rouvinen, J., Mäenpää, P.H. (1998) Putative helices 3 and 5 of the human vitamin D3 receptor are important for the binding of calcitriol. *FEBS lett. 440*, 203-207.

Van Baelen, H., Bouillon, R., De Moor, P. (1980) Vitamin D-binding protein (Gc-globulin) binds actin. *J. Biol. Chem. 255*, 2270-2272.

Verlinden, L., Verstuyf, A., Convents, R., Marcelis, S., Van Camp, M., Bouillon, R. (1998) Action of $1,25(OH)_2D_3$ on the cell cycle genes, cyclin D1, p21, and p27 in MCF-7 cells. *Mol. Cell Endocrinol. 142*, 57-65.

Veyron, P., Pamphile, R., Binderup, L., Touraine, J.L. (1993) Two novel vitamin D analogues, KH 1060 and CB 966, prolong skin allograft survival in mice. *Transpl. Immunol. 1*, 72-76.

Walters, M.R., Rosen, D.M., Norman, A.W., Luben, R.A. (1982) 1,25-Dihydroxyvitamin D receptors in an established bone cell line. Correlation with biochemical responses. *J. Biol. Chem. 257*, 7481-7484.

Wecksler, W. R., Ross, F.P., Mason, R.S., Posen, S., Norman, A.W. (1980a) Biochemical properties of the 1 alpha, 25-dihydroxyvitamin D_3 cytoplasmic receptors from human and chick parathyroid glands. *Arch. Biochem. Biophys. 201*, 95-103.

Wecksler, W. R., Ross, F.P., Mason, R.S., Norman, A.W. (1980b) Biochemical properties of the 1 alpha, 25-dihydroxyvitamin D_3 cytosol receptors from human and chicken intestinal mucosa. *J. Clin. Endocr. Metab. 50*, 152-157.

Wecksler, W.R., and Norman, A.W. (1979) An hydroxylapatite batch assay for the quantitation of 1alpha,25-dihydroxyvitamin D_3-receptor complexes. *Anal. Biochem. 92*, 314-323.

Wecksler, W.R., and Norman, A.W. (1980) A kinetic and equilibrium binding study of 1 alpha,25-dihydroxyvitamin D_3 with its cytosol receptor from chick intestinal mucosa. *J. Biol. Chem. 255*, 3571-3574.

Weisinger, J.R., Favus, M.J., Langman, C.B., Bushinsky, D.A. (1989) Regulation of 1,25-dihydroxyvitamin D_3 by calcium in the parathyroidectomized, parathyroid hormone-replete rat. *J. Bone Miner. Res. 4*, 929-935.

White, P. and Cooke, N. (2000) The multifunctional properties and characteristics of vitamin D-binding protein. *Trends Endocrinol. Metab. 11*, 320-327.

Wing, R.M., Okamura, W.H., Rego, A., Pirio, M.R., Norman, A.W. (1975) Studies on vitamin D and its analogs. VIII. Solution conformations of vitamin D_3 and 1 alpha, 25-dihydroxyvitamin D3 by high-resolution proton magnetic resonance spectroscopy. *J.*

Am. Chem. Soc. 97, 4980-4985.

Wistrom, A.C. and Meizel, S. (1993) Evidence suggesting involvement of a unique human sperm steroid receptor/Cl- channel complex in the progesterone-initiated acrosome reaction. *Dev. Biol. 159*, 679-690.

Wu, Y., Craig, T.A., Lutz, W.H., Kumar, R. (1999) Identification of 1 alpha,25-dihydroxyvitamin D_3 response elements in the human transforming growth factor beta 2 gene. *Biochemistry 38*, 2654-2660.

Yamada S., Shimizu, M., Yamamoto, K. (2003) Structure-function relationships of vitamin D including ligand recognition by the vitamin D receptor. *Med. Res. Rev. 23*, 89-115.

Yamamoto, K., Masuno, H., Choi, M., Nakashima, K., Taga, T., Ooizumi, H., Umesono, K., Sicinska, W., VanHooke, J., DeLuca, H.F. (2000) Three-dimensional modeling of and ligand docking to vitamin D receptor ligand binding domain. *Proc. Natl. Acad. Sci. USA 97*, 1467-1472.

Yamamoto, N. and Homma S. (1991) Vitamin D3 binding protein (group-specific component) is a precursor for the macrophage-activating signal factor from lysophosphatidylcholine-treated lymphocytes. *Proc. Natl. Acad. Sci. USA 88*, 8539-8543.

Zanello, L.P, and Norman, A.W. (2004) Rapid modulation of osteoblast ion channel responses by 1alpha,25$(OH)_2$-vitamin D_3 requires the presence of a functional vitamin D nuclear receptor. *Proc. Natl. Acad. Sci. USA 101*, 1589-1594.

Zeitz, U., Weber, K., Soegiarto, D.W., Wolf, E., Balling, R., Erben, R.G. (2003) Impaired insulin secretory capacity in mice lacking a functional vitamin D receptor. *FASEB J. 17*, 509-511.

Zhou, L.X., Nemere, I., Norman, A.W. (1992) 1,25-Dihydroxyvitamin D_3 analog structure-function assessment of the rapid stimulation of intestinal calcium absorption (transcaltachia). *J. Bone. Miner. Res. 7*, 457-463.

Zinser, G., Packman, K., Welsh, J. (2002) Vitamin D_3 receptor ablation alters mammary gland morphogenesis. *Development 129*, 3067-3076.

APPENDICES

Appendix A. List of Abbreviations

A	Adenine
AF	Activation Function
ATP	Adenosine Triphosphate
bp	base pair
C	Cytosine
C_i	Curies
cDNA	Complimentary Deoxyriboncleic Acid
CTP	Cytidine Triphosphate
DBD	DNA Binding Domain
DBP	DNA Binding Protein
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPM	Disintegrations per minute
DR	Direct Repeat
DTT	Dithiothreitol
EDTA	Ethylene Diamino Tetraacetic Acid
G	Guanine
GTP	Guanosine Triphosphate
HRE	Hormone Response Element
HRP	Horseradish Peroxidase

HVDRR	Hereditary vitamin D-Resistant Rickets, Type II
K_D	Equilibrium Dissociation Constant
Kb	Kilobase
kDa	Kilo Daltons
LBD	Ligand Binding Domain
LDS	Lithium Dodecyl Sulfate
mRNA	messenger Ribonucleic Acid
NCS	Newborn Calf Serum
dNTP	Deoxy-Nucleoside Triphosphate
ddNTP	Dideoxy-Nucleoside Triphosphate
PBS	Phosphate Buffered Saline
PTH	Parathyroid Hormone
RAR	Retinoic Acid Receptor
RXR	Retinoic X Receptor
T	Thymine
TBS	Tris Buffered Saline
TED	Tris-hydroxymethyl aminomethane; EDTA; DTT
dTTP	Deoxythymidine Triphosphate
TR	Thyroid Receptor
VDR	Vitamin D Receptor
VDRR I	Vitamin D-Dependent Rickets, Type I
VDRR II	Vitamin D-Dependent Rickets, Type II

Appendix B. Single Letter Abbreviations for Amino Acids

A	Alanine	**M**	Methionine
C	Cysteine	**N**	Asparagine
D	Aspartic acid	**P**	Proline
E	Glutamic acid	**Q**	Glutamine
F	Phenylalanine	**R**	Arginine
G	Glycine	**S**	Serine
H	Histidine	**T**	Threonine
I	Isoleucine	**V**	Valine
K	Lysine	**W**	Tryptophan
L	Leucine	**Y**	Tyrosine

Appendix C. Nucleotide and Amino Acid Sequence of Human VDR

National Center for Biological Information, http://www.ncbi.nih.gov, accession number J03258.

(VDR nucleotide sequence is in black, amino acid sequence is in blue, and the start and stop codons are in red and underlined.)

```
   1- GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA-  60
    -
  61- CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGATGGAG- 120
    -                                                       M  E     2
 121- GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG- 180
    - A  M  A  A  S  T  S  L  P  D  P  G  D  F  D  R  N  V  P  R    22
 181- ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA- 240
    - I  C  G  V  C  G  D  R  A  T  G  F  H  F  N  A  M  T  C  E    42
 241- GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCTTC- 300
    - G  C  K  G  F  F  R  R  S  M  K  R  K  A  L  F  T  C  P  F    62
 301- AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA- 360
    - N  G  D  C  R  I  T  K  D  N  R  R  H  C  Q  A  C  R  L  K    82
 361- CGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGG- 420
    - R  C  V  D  I  G  M  M  K  E  F  I  L  T  D  E  E  V  Q  R   102
 421- AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTGCGGCCC- 480
    - K  R  E  M  I  L  K  R  K  E  E  E  A  L  K  D  S  L  R  P   122
 481- AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCCACCATAAGACC- 540
    - K  L  S  E  E  Q  Q  R  I  I  A  I  L  L  D  A  H  H  K  T   142
 541- TACGACCCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT- 600
    - Y  D  P  T  Y  S  D  F  C  Q  F  R  P  P  V  R  V  N  D  G   162
 601- GGAGGGAGCCATCCTTCCAGGCCCAACTCCAGACACACTCCCAGCTTCTCTGGGGACTCC- 660
    - G  G  S  H  P  S  R  P  N  S  R  H  T  P  S  F  S  G  D  S   182
 661- TCCTCCTCCTGCTCAGATCACTGTATCACCTCTTCAGACATGATGGACTCGTCCAGCTTC- 720
    - S  S  S  C  S  D  H  C  I  T  S  S  D  M  M  D  S  S  S  F   202
 721- TCCAATCTGGATCTGAGTGAAGAAGATTCAGATGACCCTTCTGTGACCCTAGAGCTGTCC- 780
    - S  N  L  D  L  S  E  E  D  S  D  D  P  S  V  T  L  E  L  S   222
 781- CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT- 840
    - Q  L  S  M  L  P  H  L  A  D  L  V  S  Y  S  I  Q  K  V  I   242
 841- GGCTTTGCTAAGATGATACCAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACTG- 900
    - G  F  A  K  M  I  P  G  F  R  D  L  T  S  E  D  Q  I  V  L   262
 901- CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGTCCTTCACCATGGAC- 960
    - L  K  S  S  A  I  E  V  I  M  L  R  S  N  E  S  F  T  M  D   282
 961- GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA-1020
    - D  M  S  W  T  C  G  N  Q  D  Y  K  Y  R  V  S  D  V  T  K   302
1021- GCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG-1080
    - A  G  H  S  L  E  L  I  E  P  L  I  K  F  Q  V  G  L  K  K   322
1081- CTGAACTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT-1140
    - L  N  L  H  E  E  E  H  V  L  L  M  A  I  C  I  V  S  P  D   342
1141- CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA-1200
    - R  P  G  V  Q  D  A  A  L  I  E  A  I  Q  D  R  L  S  N  T   362
1201- CTGCAGACGTACATCCGCTGCCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAG-1260
    - L  Q  T  Y  I  R  C  R  H  P  P  P  G  S  H  L  L  Y  A  K   382
1261- ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC-1320
    - M  I  Q  K  L  A  D  L  R  S  L  N  E  E  H  S  K  Q  Y  R   402
1321- TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCCTTGTGCTCGAAGTGTTT-1380
    - C  L  S  F  Q  P  E  C  S  M  K  L  T  P  L  V  L  E  V  F   422
1381- GGCAATGAGATCTCCTGACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA-1440
    - G  N  E  I  S                                                427
```

Appendix D. Nucleotide and Amino Acid Sequence of VDR Mutants

VDR-S237A

- The start and stop codons are in red and underlined.
- Codons highlighted in yellow translate to the desired alanine mutation.

```
   1- GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA-   60
    -
  61- CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGATGGAG-  120
    -                                                       M  E      2
 121- GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG-  180
    - A  M  A  A  S  T  S  L  P  D  P  G  D  F  D  R  N  V  P  R     22
 181- ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA-  240
    - I  C  G  V  C  G  D  R  A  T  G  F  H  F  N  A  M  T  C  E     42
 241- GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCTTC-  300
    - G  C  K  G  F  F  R  R  S  M  K  R  K  A  L  F  T  C  P  F     62
 301- AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA-  360
    - N  G  D  C  R  I  T  K  D  N  R  R  H  C  Q  A  C  R  L  K     82
 361- CGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGG-  420
    - R  C  V  D  I  G  M  M  K  E  F  I  L  T  D  E  E  V  Q  R    102
 421- AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTGCGGCCC-  480
    - K  R  E  M  I  L  K  R  K  E  E  E  A  L  K  D  S  L  R  P    122
 481- AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCCACCATAAGACC-  540
    - K  L  S  E  E  Q  Q  R  I  I  A  I  L  L  D  A  H  H  K  T    142
 541- TACGACCCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT-  600
    - Y  D  P  T  Y  S  D  F  C  Q  F  R  P  P  V  R  V  N  D  G    162
 601- GGAGGGAGCCATCCTTCCAGGCCCAACTCCAGACACACTCCCAGCTTCTCTGGGGACTCC-  660
    - G  G  S  H  P  S  R  P  N  S  R  H  T  P  S  F  S  G  D  S    182
 661- TCCTCCTCCTGCTCAGATCACTGTATCACCTCTTCAGACATGATGGACTCGTCCAGCTTC-  720
    - S  S  S  C  S  D  H  C  I  T  S  S  D  M  M  D  S  S  S  F    202
 721- TCCAATCTGGATCTGAGTGAAGAAGATTCAGATGACCCTTCTGTGACCCTAGAGCTGTCC-  780
    - S  N  L  D  L  S  E  E  D  S  D  D  P  S  V  T  L  E  L  S    222
 781- CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACGCCATCCAAAAGGTCATT-  840
    - Q  L  S  M  L  P  H  L  A  D  L  V  S  Y  A  I  Q  K  V  I    242
 841- GGCTTTGCTAAGATGATACCAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACTG-  900
    - G  F  A  K  M  I  P  G  F  R  D  L  T  S  E  D  Q  I  V  L    262
 901- CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGCGCTCCAATGAGTCCTTCACCATGGAC-  960
    - L  K  S  S  A  I  E  V  I  M  L  R  S  N  E  S  F  T  M  D    282
 961- GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA-1020
    - D  M  S  W  T  C  G  N  Q  D  Y  K  Y  R  V  S  D  V  T  K    302
1021- GCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG-1080
    - A  G  H  S  L  E  L  I  E  P  L  I  K  F  Q  V  G  L  K  K    322
1081- CTGAACTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT-1140
    - L  N  L  H  E  E  E  H  V  L  L  M  A  I  C  I  V  S  P  D    342
1141- CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA-1200
    - R  P  G  V  Q  D  A  A  L  I  E  A  I  Q  D  R  L  S  N  T    362
1201- CTGCAGACGTACATCCGCTGCCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAG-1260
    - L  Q  T  Y  I  R  C  R  H  P  P  P  G  S  H  L  L  Y  A  K    382
1261- ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC-1320
    - M  I  Q  K  L  A  D  L  R  S  L  N  E  E  H  S  K  Q  Y  R    402
1321- TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCCTTGTGCTCGAAGTGTTT-1380
    - C  L  S  F  Q  P  E  C  S  M  K  L  T  P  L  V  L  E  V  F    422
1381- GGCAATGAGATCTCCTGACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA-1440
    - G  N  E  I  S                                                 427
```

VDR-R274A

- The start and stop codons are in red and underlined.
- Codons highlighted in yellow translate to the desired alanine mutation.

```
   1- GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA-  60
    -
  61- CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGATGGAG- 120
    -                                                       M  E     2
 121- GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG- 180
    - A  M  A  A  S  T  S  L  P  D  P  G  D  F  D  R  N  V  P  R    22
 181- ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA- 240
    - I  C  G  V  C  G  D  R  A  T  G  F  H  F  N  A  M  T  C  E    42
 241- GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCTTC- 300
    - G  C  K  G  F  F  R  R  S  M  K  R  K  A  L  F  T  C  P  F    62
 301- AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA- 360
    - N  G  D  C  R  I  T  K  D  N  R  R  H  C  Q  A  C  R  L  K    82
 361- CGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGG- 420
    - R  C  V  D  I  G  M  M  K  E  F  I  L  T  D  E  E  V  Q  R   102
 421- AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTGCGGCCC- 480
    - K  R  E  M  I  L  K  R  K  E  E  E  A  L  K  D  S  L  R  P   122
 481- AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCCACCATAAGACC- 540
    - K  L  S  E  E  Q  Q  R  I  I  A  I  L  L  D  A  H  H  K  T   142
 541- TACGACCCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT- 600
    - Y  D  P  T  Y  S  D  F  C  Q  F  R  P  P  V  R  V  N  D  G   162
 601- GGAGGGAGCCATCCTTCCAGGCCCAACTCCAGACACACTCCCAGCTTCTCTGGGGACTCC- 660
    - G  G  S  H  P  S  R  P  N  S  R  H  T  P  S  F  S  G  D  S   182
 661- TCCTCCTCCTGCTCAGATCACTGTATCACCTCTTCAGACATGATGGACTCGTCCAGCTTC- 720
    - S  S  S  C  S  D  H  C  I  T  S  S  D  M  M  D  S  S  S  F   202
 721- TCCAATCTGGATCTGAGTGAAGAAGATTCAGATGACCCTTCTGTGACCCTAGAGCTGTCC- 780
    - S  N  L  D  L  S  E  E  D  S  D  D  P  S  V  T  L  E  L  S   222
 781- CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACAGCATCCAAAAGGTCATT- 840
    - Q  L  S  M  L  P  H  L  A  D  L  V  S  Y  S  I  Q  K  V  I   242
 841- GGCTTTGCTAAGATGATACCAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACTG- 900
    - G  F  A  K  M  I  P  G  F  R  D  L  T  S  E  D  Q  I  V  L   262
 901- CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGGCCTCCAATGAGTCCTTCACCATGGAC- 960
    - L  K  S  S  A  I  E  V  I  M  L  A  S  N  E  S  F  T  M  D   282
 961- GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA-1020
    - D  M  S  W  T  C  G  N  Q  D  Y  K  Y  R  V  S  D  V  T  K   302
1021- GCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG-1080
    - A  G  H  S  L  E  L  I  E  P  L  I  K  F  Q  V  G  L  K  K   322
1081- CTGAACTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT-1140
    - L  N  L  H  E  E  E  H  V  L  L  M  A  I  C  I  V  S  P  D   342
1141- CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA-1200
    - R  P  G  V  Q  D  A  A  L  I  E  A  I  Q  D  R  L  S  N  T   362
1201- CTGCAGACGTACATCCGCTGCCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAG-1260
    - L  Q  T  Y  I  R  C  R  H  P  P  P  G  S  H  L  L  Y  A  K   382
1261- ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC-1320
    - M  I  Q  K  L  A  D  L  R  S  L  N  E  E  H  S  K  Q  Y  R   402
1321- TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCCTTGTGCTCGAAGTGTTT-1380
    - C  L  S  F  Q  P  E  C  S  M  K  L  T  P  L  V  L  E  V  F   422
1381- GGCAATGAGATCTCCTGACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA-1440
    - G  N  E  I  S                                                 427
```

VDR-S237A/R274A Double Mutant

- The start and stop codons are in red and underlined.
- Codons highlighted in yellow translate to the desired alanine mutation.

```
   1- GAACAGCTTGTCCACCCGCCGGCCGGACCAGAAGCCTTTGGGTCTGAAGTGTCTGTGAGA-  60
    -
  61- CCTCACAGAAGAGCACCCCTGGGCTCCACTTACCTGCCCCCTGCTCCTTCAGGGATGGAG- 120
    -                                                       M  E      2
 121- GCAATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCCGG- 180
    - A  M  A  A  S  T  S  L  P  D  P  G  D  F  D  R  N  V  P  R     22
 181- ATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGACCTGTGAA- 240
    - I  C  G  V  C  G  D  R  A  T  G  F  H  F  N  A  M  T  C  E     42
 241- GGCTGCAAAGGCTTCTTCAGGCGAAGCATGAAGCGGAAGGCACTATTCACCTGCCCCTTC- 300
    - G  C  K  G  F  F  R  R  S  M  K  R  K  A  L  F  T  C  P  F     62
 301- AACGGGGACTGCCGCATCACCAAGGACAACCGACGCCACTGCCAGGCCTGCCGGCTCAAA- 360
    - N  G  D  C  R  I  T  K  D  N  R  R  H  C  Q  A  C  R  L  K     82
 361- CGCTGTGTGGACATCGGCATGATGAAGGAGTTCATTCTGACAGATGAGGAAGTGCAGAGG- 420
    - R  C  V  D  I  G  M  M  K  E  F  I  L  T  D  E  E  V  Q  R    102
 421- AAGCGGGAGATGATCCTGAAGCGGAAGGAGGAGGAGGCCTTGAAGGACAGTCTGCGGCCC- 480
    - K  R  E  M  I  L  K  R  K  E  E  E  A  L  K  D  S  L  R  P    122
 481- AAGCTGTCTGAGGAGCAGCAGCGCATCATTGCCATACTGCTGGACGCCCACCATAAGACC- 540
    - K  L  S  E  E  Q  Q  R  I  I  A  I  L  L  D  A  H  H  K  T    142
 541- TACGACCCCACCTACTCCGACTTCTGCCAGTTCCGGCCTCCAGTTCGTGTGAATGATGGT- 600
    - Y  D  P  T  Y  S  D  F  C  Q  F  R  P  P  V  R  V  N  D  G    162
 601- GGAGGGAGCCATCCTTCCAGGCCCAACTCCAGACACACTCCCAGCTTCTCTGGGGACTCC- 660
    - G  G  S  H  P  S  R  P  N  S  R  H  T  P  S  F  S  G  D  S    182
 661- TCCTCCTCCTGCTCAGATCACTGTATCACCTCTTCAGACATGATGGACTCGTCCAGCTTC- 720
    - S  S  S  C  S  D  H  C  I  T  S  S  D  M  M  D  S  S  S  F    202
 721- TCCAATCTGGATCTGAGTGAAGAAGATTCAGATGACCCTTCTGTGACCCTAGAGCTGTCC- 780
    - S  N  L  D  L  S  E  E  D  S  D  D  P  S  V  T  L  E  L  S    222
 781- CAGCTCTCCATGCTGCCCCACCTGGCTGACCTGGTCAGTTACGCCATCCAAAAGGTCATT- 840
    - Q  L  S  M  L  P  H  L  A  D  L  V  S  Y  A  I  Q  K  V  I    242
 841- GGCTTTGCTAAGATGATACCAGGATTCAGAGACCTCACCTCTGAGGACCAGATCGTACTG- 900
    - G  F  A  K  M  I  P  G  F  R  D  L  T  S  E  D  Q  I  V  L    262
 901- CTGAAGTCAAGTGCCATTGAGGTCATCATGTTGGCCTCCAATGAGTCCTTCACCATGGAC- 960
    - L  K  S  S  A  I  E  V  I  M  L  A  S  N  E  S  F  T  M  D    282
 961- GACATGTCCTGGACCTGTGGCAACCAAGACTACAAGTACCGCGTCAGTGACGTGACCAAA-1020
    - D  M  S  W  T  C  G  N  Q  D  Y  K  Y  R  V  S  D  V  T  K    302
1021- GCCGGACACAGCCTGGAGCTGATTGAGCCCCTCATCAAGTTCCAGGTGGGACTGAAGAAG-1080
    - A  G  H  S  L  E  L  I  E  P  L  I  K  F  Q  V  G  L  K  K    322
1081- CTGAACTTGCATGAGGAGGAGCATGTCCTGCTCATGGCCATCTGCATCGTCTCCCCAGAT-1140
    - L  N  L  H  E  E  E  H  V  L  L  M  A  I  C  I  V  S  P  D    342
1141- CGTCCTGGGGTGCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACA-1200
    - R  P  G  V  Q  D  A  A  L  I  E  A  I  Q  D  R  L  S  N  T    362
1201- CTGCAGACGTACATCCGCTGCCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAG-1260
    - L  Q  T  Y  I  R  C  R  H  P  P  P  G  S  H  L  L  Y  A  K    382
1261- ATGATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTACCGC-1320
    - M  I  Q  K  L  A  D  L  R  S  L  N  E  E  H  S  K  Q  Y  R    402
1321- TGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCCTTGTGCTCGAAGTGTTT-1380
    - C  L  S  F  Q  P  E  C  S  M  K  L  T  P  L  V  L  E  V  F    422
1381- GGCAATGAGATCTCCTGACTAGGACAGCCTGTGCGGTGCCTGGGTGGGGCTGCTCCTCCA-1440
    - G  N  E  I  S                                                 427
```

APPENDIX E. Binding Assay Raw Data in DPM with FreeStyle™ 293-F Cells

Wild-type VDR

Trial 1:

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
6.510	7015.06	5798.99	6428.84	6414.3	281.47	247.49	264.48	6149.82
3.505	5534.05	5251.87	5006.48	5264.13	122.99	123.15	123.07	5141.06
2.011	3155.38	3352.23	3026.97	3178.19	69.21	77.37	73.29	3104.9
0.707	1336.64	1223.76	1269.86	1276.75	48.25	42.02	45.14	1231.62
0.287	700.82	725.99	619.61	682.14	31.83	40.77	36.3	645.84
0.196	186.7	365.7	365.28	305.89	31.71	32.87	32.29	273.6

Trial 2:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.510	3754.72	4083.21	3794.63	3877.52	243.3	193.28	218.29	3659.23
3.505	3390.7	3722.42	3627.11	3580.08	102.15	135.82	118.99	3461.09
2.011	2504.56	2839.6	2473	2605.72	70.08	66.68	68.38	2537.34
0.707	972.04	1108.42	1058.23	1046.23	44.45	28.97	36.71	1009.52
0.287	551.05	700.93	531.1	594.36	33.87	27.18	30.53	563.84
0.196	224.73	301.26	312.61	279.53	26.72	54.08	40.4	239.13

Trial 3:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
7.607	2885.46	3195.12	3142.49	3074.36	260.73	268.57	264.65	2809.71
4.925	2905.62	2814.69	2912.02	2877.44	166.75	150.95	158.85	2718.59
2.611	2780.35	2789.57	3015.48	2861.8	87.64	116.87	102.255	2759.55
1.582	1370.54	2072.39	2102.02	1848.32	60.5	67.76	64.13	1784.19
0.546	808.56	841.36	877.41	842.44	39.04	47.7	43.37	799.07
0.261	370.93	414	436.16	407.03	34.45	26.62	30.535	376.5
0.133	186.66	225.07	237.54	216.423	22.59	27.54	25.065	191.35

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

Wild-type VDR (Cont'd)

Trial 4:

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
5.354	2000.7	2043.15	1779.51	1941.12	160.09	160.09	160.09	1781.03
2.850	1662.35	1728.09	1509.5	1633.31	110.23	95.17	102.7	1530.61
1.580	1425.51	1596.55	1471.63	1497.9	50.97	269.8	160.39	1337.51
0.554	860.38	902.16	689.02	817.187	59.32	88.75	74.04	743.15
0.250	417.99	514.18	353.69	428.62	41.72	30.91	36.32	392.31
0.123	110.32	152.69	206.48	156.497	31.16	27.72	29.44	127.06

Trial 5:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.648	6406.3	5297.34	5361.29	5688.31	460.98	501.42	481.2	5207.11
3.478	4779.61	4889.65	3664.65	4444.64	315.1	421.33	368.22	4076.42
1.704	3170.67	3808.81	3800.37	3593.28	197.6	186.93	192.27	3401.02
0.705	1315.9	1618.86	1444.73	1459.83	158.77	180.83	169.8	1290.03
0.327	767.59	1310.16	756.7	944.817	221.62	77.15	149.39	795.43
0.177	522.96	610.66	474.49	536.037	102.66	81.15	91.91	444.13

* *Concentration of $[^3H]$-1α,25(OH)$_2$D$_3$ in nM*

VDR-S237A

Trial 1:

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
6.648	2216.75	1732.73	1934.39	1961.29	438.29	388.25	413.27	1548.02
3.478	1117.41	1300.79	1431.61	1283.27	291.25	261.16	276.205	1007.07
1.704	758.28	771.1	846.39	791.92	181.25	183.83	182.54	609.38
0.705	375.92	463.69	371.44	403.68	279.04	194.62	236.83	166.85
0.327	367.15	232.11	284.53	294.59	103.17	127.38	115.275	179.32
0.177	317.84	204.72	187.26	236.61	118.97	74.28	96.625	139.98

Trial 2:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.510	936.12	986.68	924.42	949.07	133.34	147.4	140.37	808.70
3.505	846.52	856.67	777.48	826.89	158.59	96.83	127.71	699.18
2.011	649.93	566.52	629.34	615.26	57.88	61.6	59.74	555.52
0.707	226.24	238.23	226.25	230.24	37.84	25.26	31.55	198.69
0.287	169.37	192.72	142.64	168.24	23.53	32.83	28.18	140.06
0.196	88.19	105.17	92.03	95.13	45.65	21.9	33.78	61.36

Trial 3:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.51	1101.97	942.89	918.19	987.68	91.22	137.03	114.13	873.56
3.51	775.69	740.18	737.29	751.05	87.75	92.71	90.23	660.82
2.01	476.28	518.50	574.32	523.03	75.98	66.43	71.21	451.83
0.71	149.81	221.82	283.68	218.44	32.10	30.02	31.06	187.38
0.29	140.11	138.34	138.07	138.84	22.76	26.20	24.48	114.36
0.20	83.70	137.83	97.31	106.28	22.94	18.08	20.51	85.77

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

VDR-S237A (Cont'd)

Trial 4:

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
5.35	1962.69	2071.24	1949.96	1994.63	160.86	145.88	153.37	1841.26
2.85	1204.10	1330.86	1643.66	1392.87	132.28	112.02	122.15	1270.72
1.58	680.55	842.52	602.93	708.67	226.20	113.39	169.80	538.87
0.55	253.40	266.67	271.05	263.71	52.85	39.88	46.37	217.34
0.25	150.45	139.58	166.55	152.19	25.55	45.95	35.75	116.44
0.12	58.42	88.69	84.03	77.05	22.94	32.33	27.64	49.41

* *Concentration of $[^3H]$-1α,25(OH)$_2$D$_3$ in nM*

VDR-R274A

Trial 1:

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
6.65	476.86	460.28	483.47	473.54	368.40	316.83	342.62	130.92
3.48	315.27	424.06	476.45	405.26	173.35	392.45	282.90	122.36
1.70	201.33	180.95	269.98	217.42	93.81	165.93	129.87	87.55
0.71	95.53	87.50	86.74	89.92	46.39	73.42	59.91	30.02
0.33	70.26	57.14	59.89	62.43	47.20	31.91	39.56	22.88
0.18	44.06	50.07	41.85	45.33	31.82	29.24	30.53	14.80

Trial 2:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.51	191.78	222.73	214.78	209.76	153.51	179.78	166.65	43.12
3.51	108.52	110.27	135.67	118.15	100.35	89.87	95.11	23.04
2.01	67.38	93.37	67.24	76.00	51.18	80.59	65.89	10.11
0.71	49.25	48.93	41.62	46.60	30.81	32.80	31.81	14.80
0.29	38.68	42.46	40.57	40.57	28.13	25.50	26.82	13.76
0.20	31.63	31.57	31.69	31.63	33.39	25.18	29.29	2.35

Trial 3:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.51	453.67	240.47	272.15	322.10	152.84	159.35	156.10	166.00
3.51	300.00	168.69	182.82	217.17	251.75	208.94	230.35	-13.18
2.01	93.46	170.00	125.44	129.63	76.69	61.05	68.87	60.76
0.71	62.25	72.33	59.67	64.75	58.03	29.86	43.95	20.81
0.29	49.83	39.83	39.87	43.18	27.17	34.34	30.76	12.42
0.20	34.25	25.43	39.84	33.17	26.54	31.81	29.18	4.00

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

VDR-S237A/R274A Double Mutant

Trial 1:

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
6.51	239.45	166.88	173.94	193.42	186.83	237.98	212.41	-18.98
3.51	108.29	98.86	97.34	101.50	90.75	75.84	83.30	18.20
2.01	61.38	59.36	25.22	48.65	50.77	56.01	53.39	-4.74
0.71	37.36	36.32	35.28	36.32	40.87	32.51	36.69	-0.37
0.29	31.83	33.40	34.96	33.40	20.02	25.33	22.68	10.72
0.20	27.84	25.20	27.86	26.97	26.16	20.72	23.44	3.53

Trial 2:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.51	190.23	163.98	179.29	177.83	175.35	173.17	174.26	3.57
3.51	100.31	110.60	116.91	109.27	86.78	74.90	80.84	28.43
2.01	74.38	73.52	70.20	72.70	54.80	43.89	49.35	23.36
0.71	38.08	39.63	50.33	42.68	37.42	24.63	31.03	11.66
0.29	28.76	35.36	34.79	32.97	26.15	30.59	28.37	4.60
0.20	24.28	45.07	34.15	34.50	27.12	27.95	27.54	6.97

Trial 3:

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.65	308.68	305.40	368.62	327.57	617.59	283.21	450.4	-122.83
3.48	197.25	195.44	627.05	339.91	187.49	175.83	181.66	158.25
1.70	181.79	110.15	118.47	136.80	312.23	140.39	226.31	-89.51
0.71	74.39	53.45	82.98	70.27	46.65	34.60	40.625	29.65
0.33	46.76	48.54	48.64	47.98	45.39	38.53	41.96	6.02
0.18	29.16	35.54	29.40	31.37	22.73	21.63	22.18	9.19

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

APPENDIX F. Binding Assay Raw Data in DPM with COS-7 Cells

Wild-type VDR

Trial 1 (at room temperature):

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
5.72	842.18	262.26	400.74	501.73	351.95	343.42	347.69	154.04
3.11	385.54	285.61	268.27	313.14	161.86	150.49	156.18	156.97
1.26	207.85	195.08	212.05	204.99	147.73	84.28	116.01	88.99
0.60	115.21	142.19	123.04	126.81	60.39	79.20	69.80	57.02
0.30	109.87	103.04	111.62	108.18	32.26	44.80	38.53	69.65
0.16	93.85	80.72	82.44	85.67	36.93	33.19	35.06	50.61

Trial 2 (at 4°C):

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.75	393.35	445.90	402.56	413.94	278.22	283.20	280.71	133.23
3.48	361.22	332.07	342.58	345.29	144.07	174.72	159.40	185.90
2.07	219.76	322.74	265.72	269.41	110.38	112.75	111.57	157.84
0.65	160.55	176.37	172.91	169.94	53.57	59.44	56.51	113.44
0.35	137.31	210.37	164.82	170.83	44.48	55.40	49.94	120.89
0.18	95.30	94.93	71.02	87.08	82.89	46.36	64.63	22.46

Trial 3 (at 4°C)

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
4.38	425.03	346.64	295.22	355.63	170.39	180.83	175.61	180.02
2.66	190.70	248.33	244.74	227.92	132.59	110.32	121.46	106.47
1.63	134.96	131.47	206.12	157.52	73.34	64.09	68.72	88.80
0.57	89.10	69.00	82.81	80.30	30.02	20.07	25.05	55.26
0.26	46.48	76.72	70.46	64.55	26.36	21.23	23.80	40.76
0.16	35.32	34.18	35.47	34.99	16.75	20.43	18.59	16.40

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

VDR-S237A

Trial 1 (at room temperature):

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
5.72	312.12	497.12	383.47	397.57	283.88	248.09	265.99	131.59
3.11	290.55	309.32	293.72	297.86	133.20	116.82	125.01	172.85
1.26	209.21	243.26	256.24	236.24	111.42	159.62	135.52	100.72
0.60	131.25	142.50	201.62	158.46	60.41	32.79	46.60	111.86
0.30	126.92	103.41	178.09	136.14	100.65	34.44	67.55	68.60
0.16	65.93	120.29	92.48	92.90	26.24	47.65	36.95	55.96

Trial 2 (at 4°C):

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
5.50	488.48	422.99	353.49	421.65	251.03	240.13	245.58	176.07
2.46	262.97	272.55	243.51	259.68	198.20	141.33	169.77	89.91
1.42	397.41	191.29	200.29	263.00	119.78	103.23	111.51	151.49
0.55	107.02	104.00	120.63	110.55	60.27	63.26	61.77	48.79
0.23	74.93	67.94	76.44	73.10	31.03	38.12	34.58	38.53
0.13	54.78	44.01	45.18	47.99	36.27	45.28	40.78	7.22

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

VDR-R274A

Trial 1 (at room temperature):

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
3.11	268.04	289.06	248.12	268.41	149.35	153.75	151.55	116.86
1.26	183.79	193.29	260.03	212.37	122.55	107.04	114.80	97.58
0.60	141.01	127.38	129.56	132.65	38.89	46.23	42.56	90.09
0.30	133.27	112.46	118.26	121.33	38.06	46.42	42.24	79.09
0.16	66.40	83.54	96.25	82.06	35.07	26.03	30.55	51.51

Trial 2 (at room temperature):

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.75	484.49	485.57	449.81	473.29	240.10	280.80	260.45	212.84
3.48	342.78	420.74	350.72	371.41	177.28	268.04	222.66	148.75
2.07	267.22	403.66	298.89	323.26	109.28	136.55	122.92	200.34
0.65	161.06	181.56	136.93	159.85	70.59	44.68	57.64	102.22
0.35	163.49	171.06	170.65	168.40	40.32	31.36	35.84	132.56
0.18	78.48	86.27	105.46	90.07	38.40	61.62	50.01	40.06

Trial 3 (at room temperature):

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
6.75	381.60	399.68	461.60	414.29	235.66	398.99	317.33	96.97
3.48	343.35	313.73	292.63	316.57	186.25	225.65	205.95	110.62
2.07	245.21	226.43	269.62	247.09	105.13	138.56	121.85	125.24
0.65	176.66	190.05	156.15	174.29	76.80	70.76	73.78	100.51
0.35	138.62	168.94	120.82	142.79	40.94	53.01	46.98	95.82
0.18	110.58	102.69	82.54	98.60	35.93	28.69	32.31	66.29

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

VDR-S237A/R274A Double Mutant

Trial 1 (at room temperature):

nM*	Total binding			Average	Nonspecific binding		Average	Specific binding
12.08	501.29	499.35	680.50	560.38	450.44	437.63	444.04	116.35
6.09	407.77	317.46	307.77	344.33	253.34	231.17	242.26	102.08
3.34	230.05	326.45	314.31	290.27	186.66	188.78	187.72	102.55
1.33	138.08	148.91	150.09	145.69	55.62	87.45	71.54	74.16
0.60	129.71	113.96	92.73	112.13	37.91	47.05	42.48	69.65
0.29	82.24	75.07	86.52	81.28	59.75	36.65	48.20	33.08

Trial 2 (at room temperature):

nM	Total binding			Average	Nonspecific binding		Average	Specific binding
5.27	393.41	464.60	413.88	423.96	475.07	319.65	397.36	26.60
2.73	259.48	246.06	415.45	307.00	153.67	171.51	162.59	144.41
1.44	188.48	240.34	195.88	208.23	93.51	119.73	106.62	101.61
0.49	144.91	143.75	131.96	140.21	53.28	77.32	65.30	74.91
0.22	122.43	127.94	115.65	122.01	32.06	30.28	31.17	90.84
0.12	104.42	95.44	85.79	95.22	69.90	31.97	50.94	44.28

Trial 3 (at 4°C):

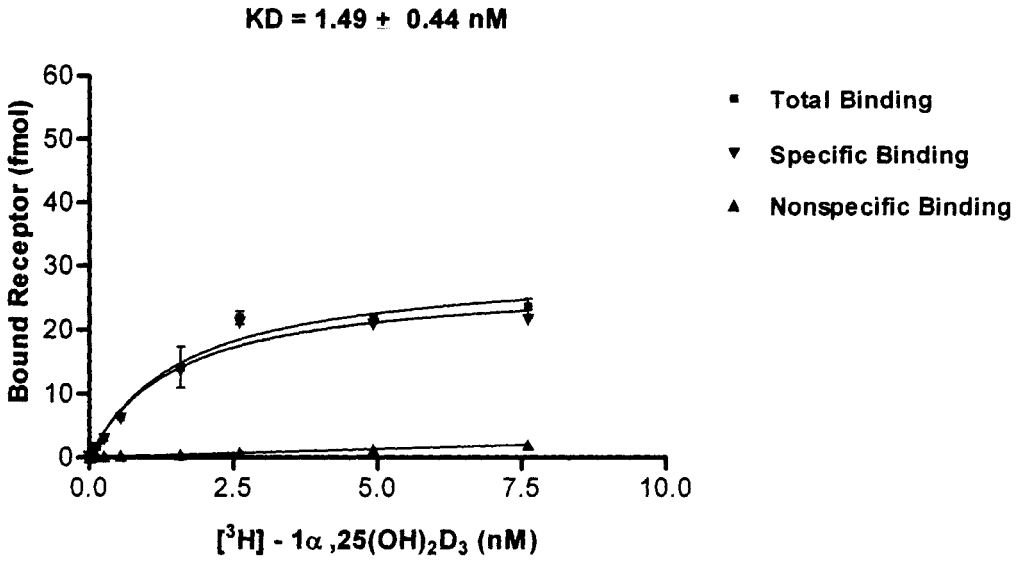
nM	Total binding			Average	Nonspecific binding		Average	Specific binding
5.50	272.80	344.07	427.02	347.96	296.03	196.92	246.48	101.49
2.46	281.49	257.82	173.55	237.62	218.67	151.99	185.33	52.29
1.42	256.48	152.12	186.39	198.33	100.43	228.10	164.27	34.07
0.55	83.80	94.49	93.23	90.51	58.68	51.97	55.33	35.18
0.23	79.28	79.41	60.52	73.07	46.78	43.13	44.96	28.12
0.13	68.32	58.69	31.98	53.00	37.21	32.76	34.99	18.01

* *Concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ in nM*

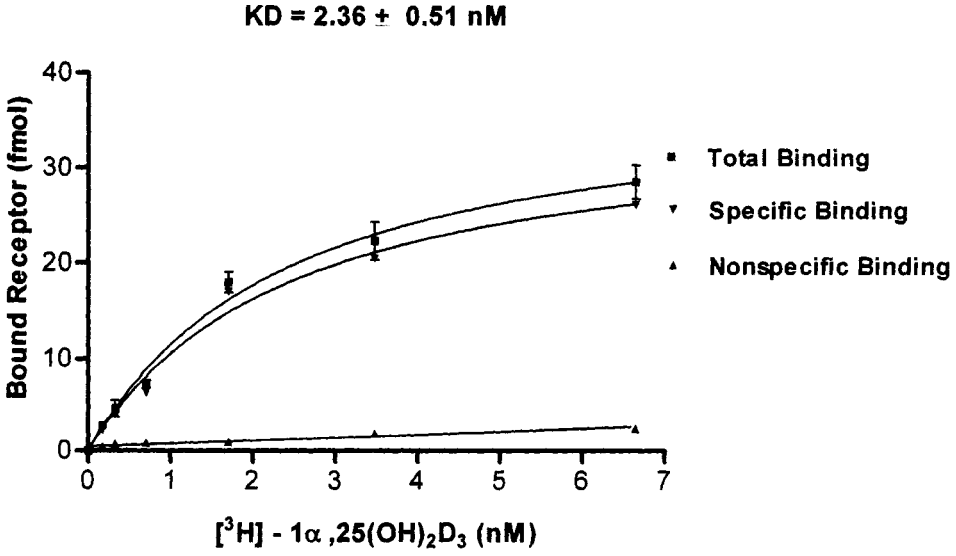
APPENDIX G. Binding Assay Curves with FreeStyle™ 293-F Cells

Wild-type VDR

Trial 1:

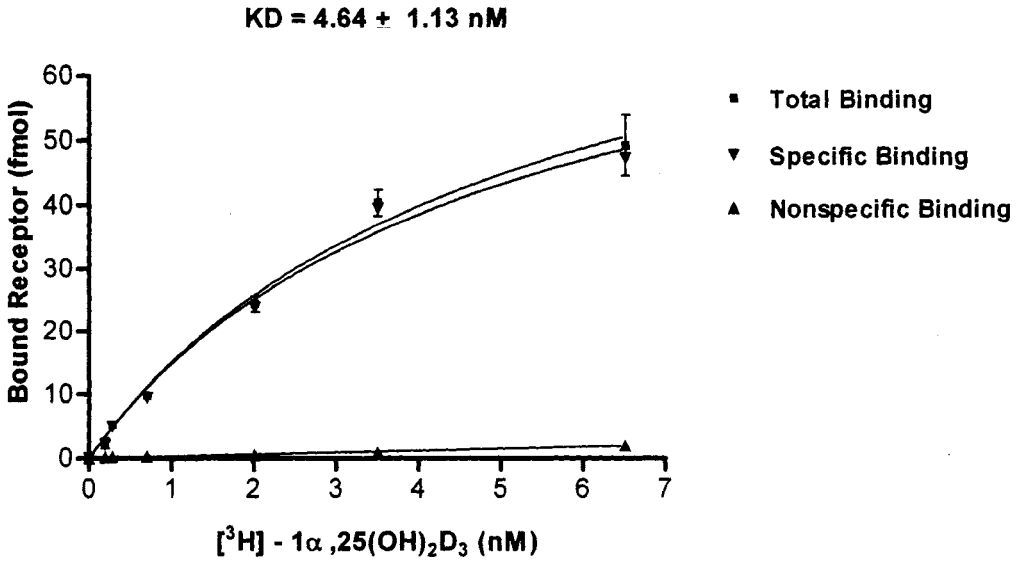


Trial 2:

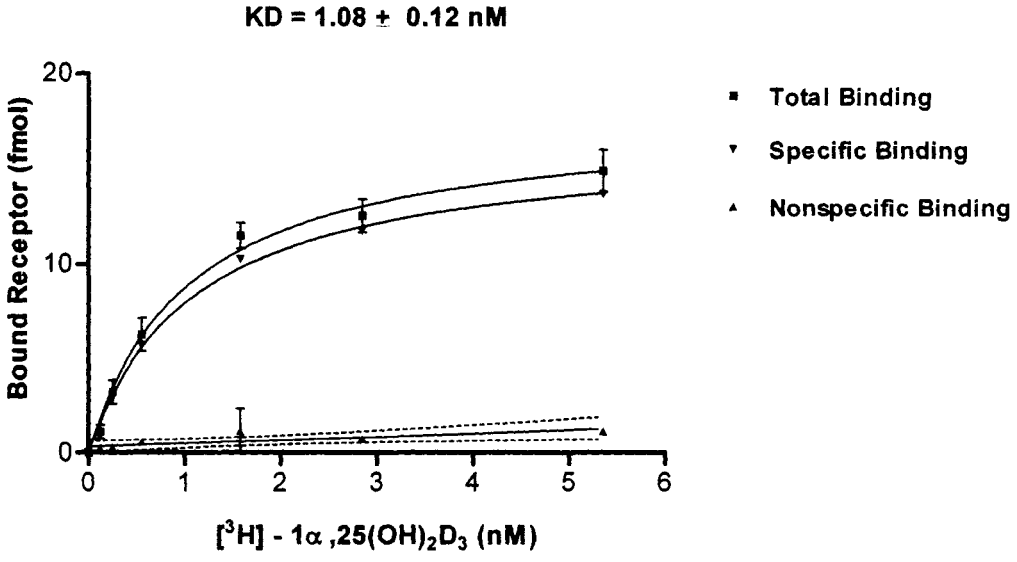


Wild-type VDR (Cont'd)

Trial 3:

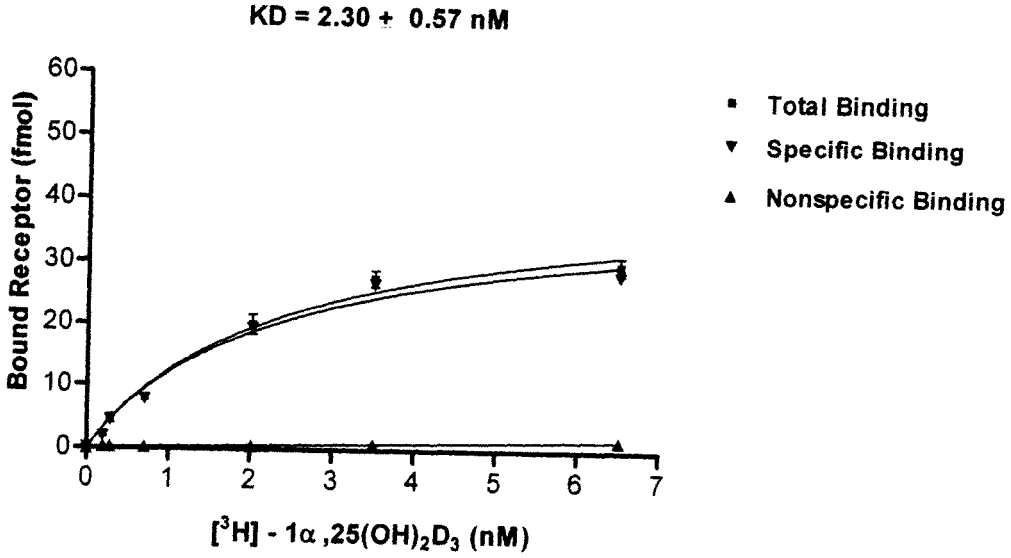


Trial 4:



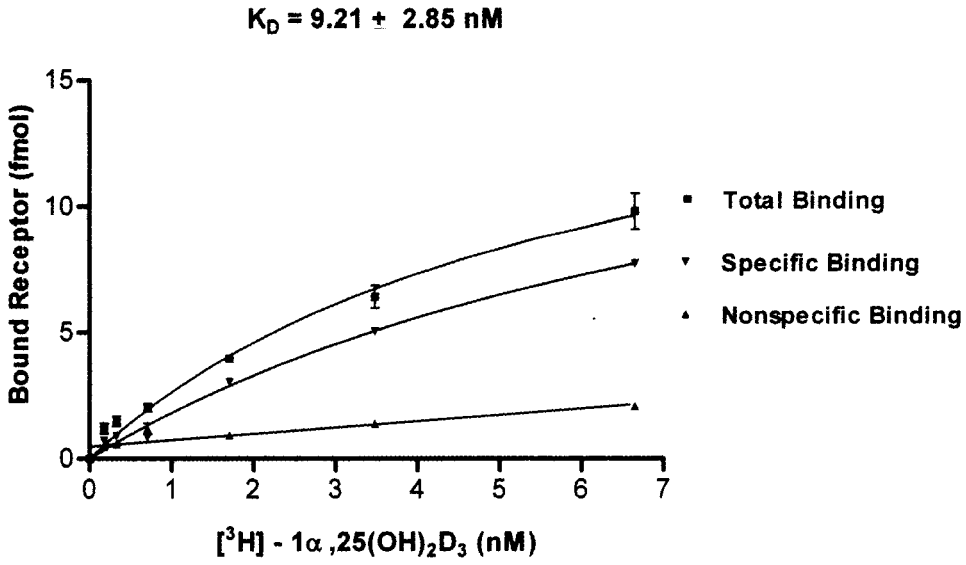
Wild-type VDR (Cont'd)

Trial 5:

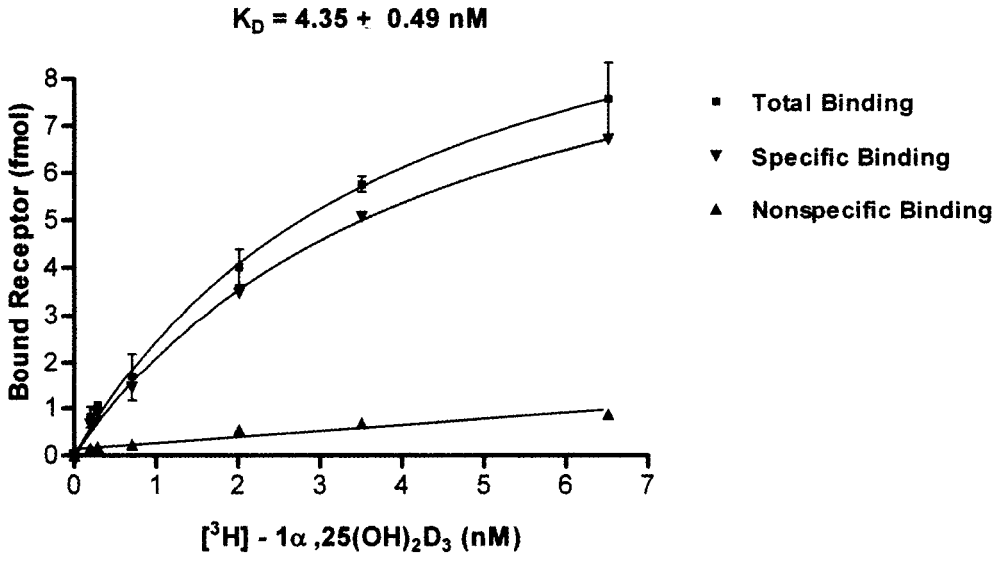


VDR-S237A

Trial 1:

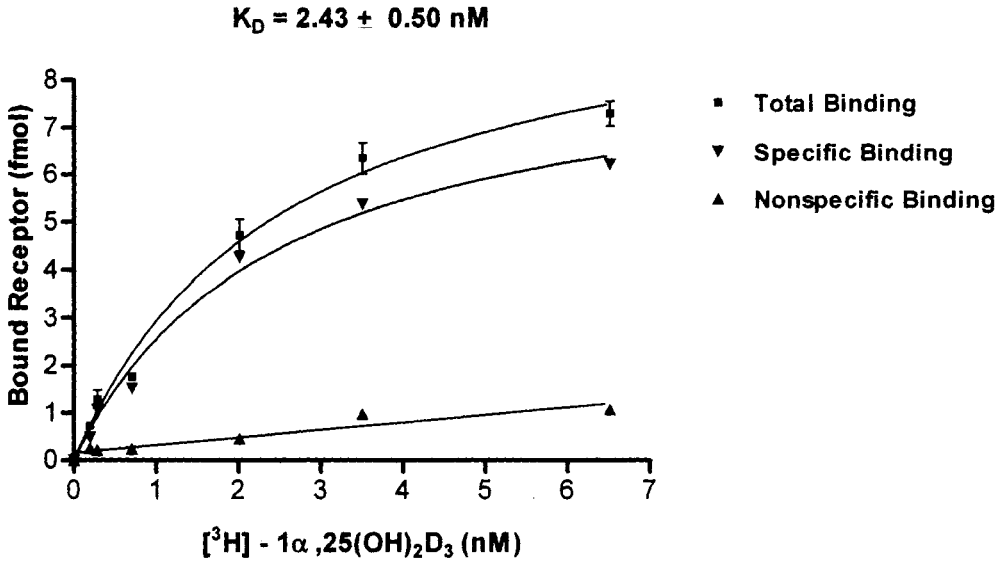


Trial 2:

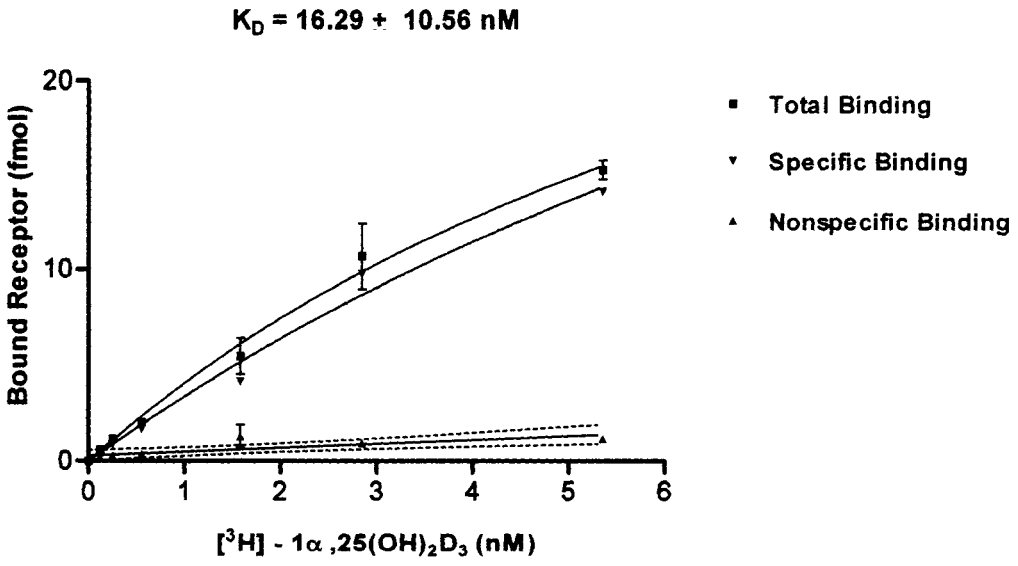


VDR-S237A (Cont'd)

Trial 3:

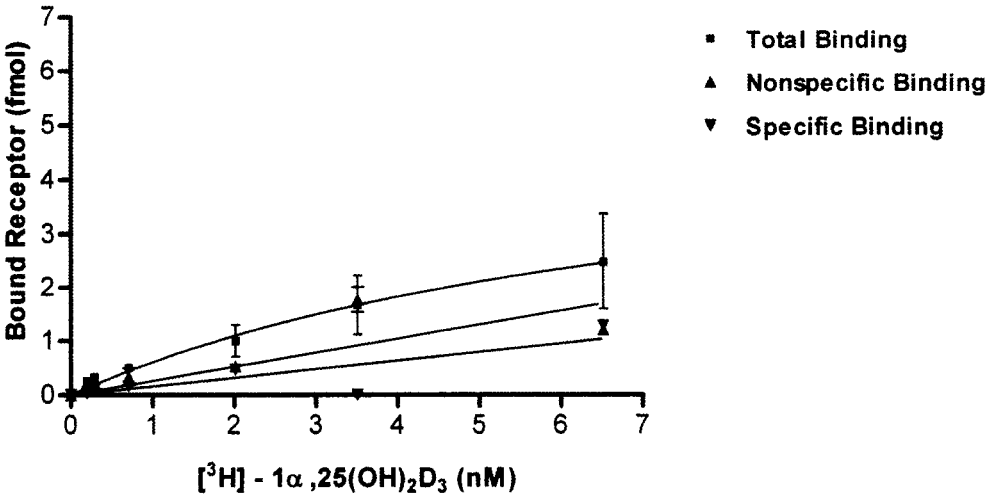


Trial 4:

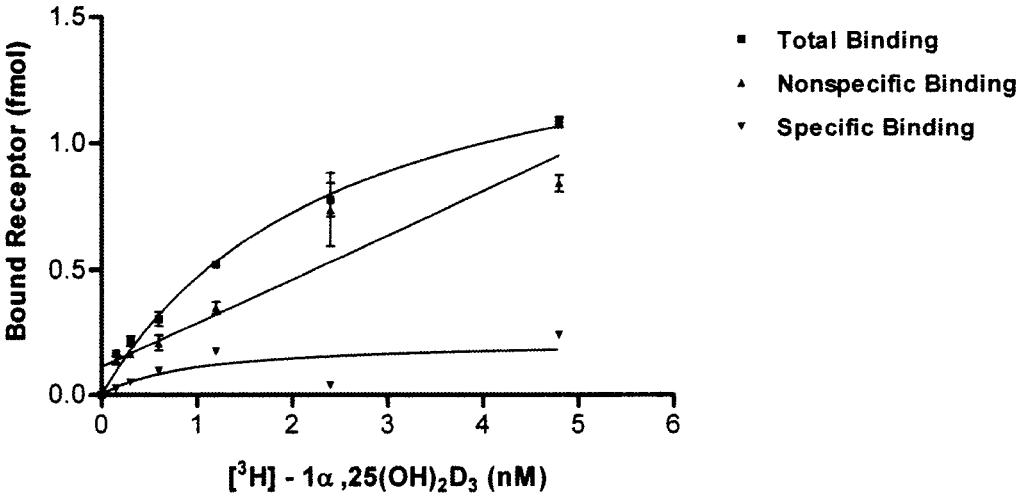


VDR-R274A

Trial 1:

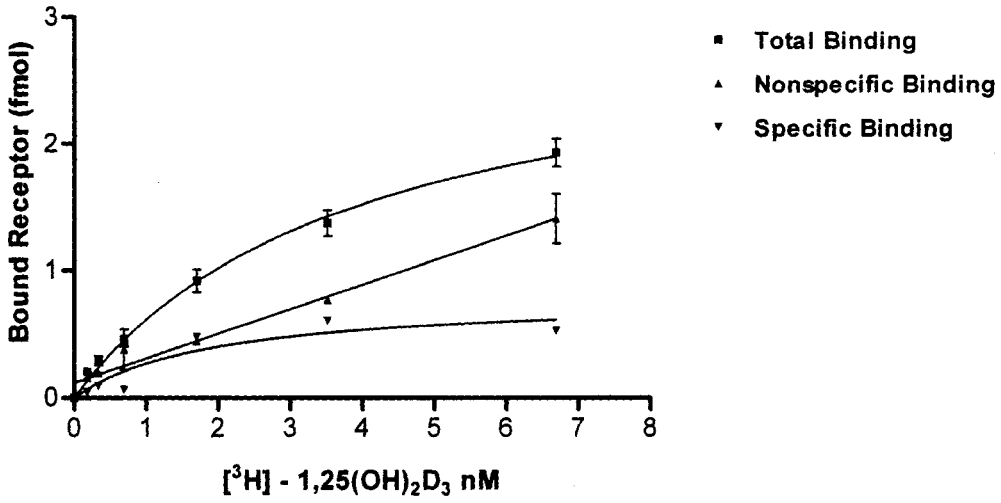


Trial 2:



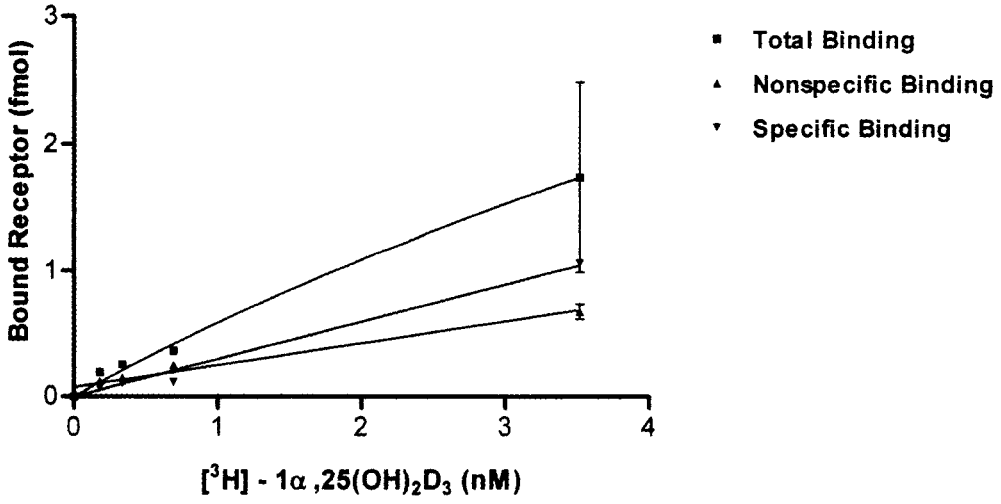
VDR-R274A (Cont'd)

Trial 3:

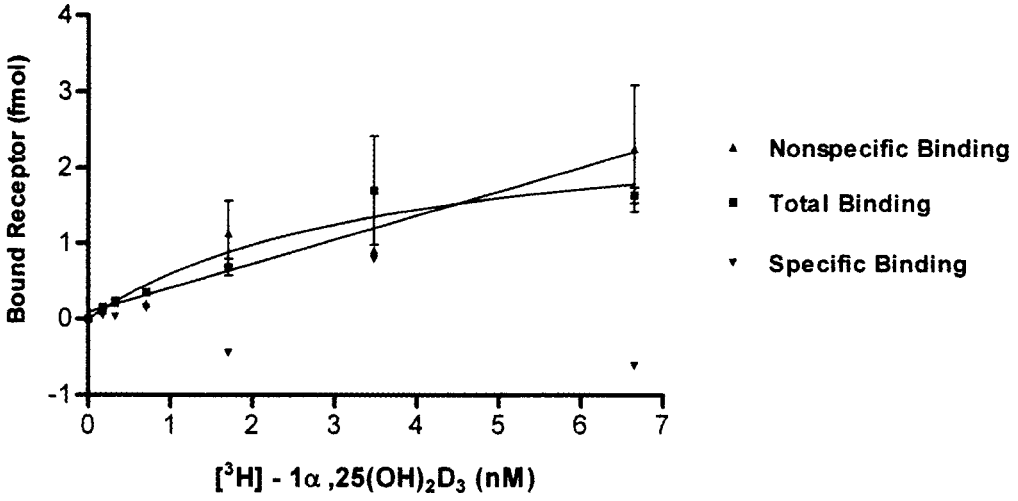


VDR-S237A/R274A Double Mutant

Trial 1:



Trial 2:



VDR-S237A/R274A Double Mutant (Cont'd)

Trial 3: